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RELIABILITY OF FERMENTATION TESTS IN IDENTIFICATION OF THE MONILIAS*

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THE constancy in fermentation of sugars by the monilias is still controversial. One may find in the literature complete rejection of sugar fermentations or complete acceptance of retained ability to ferment certain sugars over a period of years.

Castellani earlier proposed that the monilias be divided into species on the basis of their fermentation reactions, but later came to regard such reactions as by no means constant.[1] He stressed the importance of this determination as soon as possible after isolation. Ashford,[2] Garrod and Stone,[3] and Benham[4, 5] considered this method as not reliable for the classification of these organisms. Wachowiak and coworkers[6] were unable to obtain consistent fermentation of sugars in simultaneous tests or in tests repeated over a period of years. Lamb and Lamb[7] have expressed dissatisfaction with the usual method of determining sugars fermented by monilia and proposed chemical tests for sugar consumed as conclusive evidence of fermentation.

On the other hand, Fineman[8] and others have stated that the monilias may be classified by their fermentation reactions, and that their ability to ferment given sugars is retained over a considerable period of time. Stovall and coworkers[9-13] presented evidence that the monilias which they have isolated, as well as those obtained from culture collections, may be separated into three readily distinguishable groups, using this method and physiologic criteria.

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EXPERIMENTATION

This report contains our observations on and an explanation of the variability in the fermentation reactions in monilias isolated from cases of oral thrush and vulvovaginitis. It has been established positively by Hesseltine[14] and Plass and coworkers[15, 16] that these organisms are clinically significant. The methods of classification of these organisms are discussed in another paper.[17]

The fact that one or more replicate tubes of the same sugar broth occasionally failed to show the production of gas led to a consideration of at least one of the factors operative in the inconstant fermentation characters reported for the monilias by some workers. Near the beginning of the work with these organisms, fermentation tests were run in triplicate in sugar broths with bromcresol purple and bromthymol blue as indicators. Except for the greater sensitivity of the bromthymol blue to changes in acidity, the results were identical.

The sugars were used in 1 per cent concentration in a broth containing: peptone 1.0 per cent, beef extract 0.35 per cent, and sodium chloride 0.5 per cent. Bromcresol purple was the regular indicator. The milk was made up by resuspending 90 gm. of skimmed milk powder in a liter of water, adding bromcresol purple in a concentration of 0.005 per cent,[18] tubing, and sterilizing by heating for thirty minutes in an Arnold sterilizer on three successive days. After cooling, calcium lactate solution was added as given by Stovall and Bubolz.[11]

Incubation was at 37° C. and observations were made on the second, third, fourth, and seventh days.

In the tests in which bromcresol purple was compared as an indicator with bromthymol blue, one or more of the six tubes of each sugar broth occasionally failed to show production of gas. The cultures showing such variations are reported in Table I. Out of 20 strains tested, 10 failed to produce gas in one or more tubes with one or more different sugars. The failure to produce gas occurred most frequently in those sugars which were not readily fermented.

TABLE I

FAILURE OF GAS PRODUCTION BY MONILIA IN REPLICATE FERMENTATIONS

CULTURE NUMBER	SUGARS			
	LEVULOSE	MANNOSE	GALACTOSE	MALTOSE
	TUBES OUT OF 6 SHOWING FAILURE OF GAS PRODUCTION			
262	1	3	Not fermented	0
262A	1	3	5	0
263	2	2	Not fermented	0
266	0	2	Not fermented	0
268	0	0	4	1
270	0	0	4	0
271	0	0	2	0
274	0	2	5	0
275	0	1	Not fermented	0
276	0	5	Not fermented	0

Glucose, the most easily fermented of the sugars tested, gave no failures. Maltose, which is fermented almost as readily as glucose, failed to produce gas in only one tube out of 120. In decreasing order of fermentability are levulose, mannose, and galactose. Galactose is fermented poorly, and was the most inconstant in gas production.

Several strains which had been used in earlier work were run through the fermentation tests. The results differed from those determined by another observer. Two of the most variable strains, 104 and 155, were inoculated into a series of the sugar broths by three persons. The inoculum was from tubes of parallel transfers on Sabouraud's agar. Table II gives the results of these tests. It is apparent that quite different results may be obtained by three persons working with the same strain, using the same batch of sugar broths, incubating for the same length of time, and making observations at similar intervals. The personal equation in reading the results was eliminated by having the readings checked by one person (C). The differences obtained in this test were believed to be due to a variable amount of inoculum. Accordingly, several tests were made using a large and a small inoculum.

The cultures selected for the experiments with variable amounts of inoculum were, except 56924 and 6508—0.30, those which had shown inconstant fermentation reactions in previous tests. Strains 303 and 305 were identified as *Monilia albicans*, Strains 306 and 6508—0.30 as *Monilia candida*, strain 56924 is an endomyces,* and 155 is an unidentified monilia, while Strains 300 and 304 were like *Monilia albicans* in most respects and probably belong to that species. The inoculum used was from an approximately forty-eight-hour growth on a Sabouraud's glucose agar slant. The small inoculum filled only a very small arc of the circle of a 2 mm. loop, while the large inoculum nearly filled it. Table III gives the results of this test. Gas appeared later and in smaller amounts in those tubes with the small inoculum than in those with the large inoculum. No gas was formed in some cases when a small inoculum was used. In two instances gas was produced when a small but not when a large inoculum was used.

Essentially the same test was performed with the inoculum grown in glucose broth for forty-eight hours. A 0.05 c.c. and a 0.5 c.c. portion of the broth suspension of the organisms was used for the small and the large inoculum, respectively. This source of inoculum appeared to be more uniform in that there was no greater change produced by the small than the large inoculum. As in the previous test, gas appeared earlier and in greater amounts when a larger portion was used, and with some strains gas was formed by the large inoculum only. Controls were run with distilled water and peptone broth containing fermentation tubes, in order to check whether gas was formed by any unused sugar remaining in the inoculum. No gas was formed in these controls. Another thought was that the large inoculum might introduce a sufficient number of cells with stored food reserves that gas could be produced from this source. No gas was formed in the absence of fermentable sugars by the organism from either the glucose broth suspensions or Sabouraud's glucose agar slants.

The data presented in Tables II, III, and IV indicate that the size of inoculum is apparently a factor in the variability of the fermentation reactions given by the monilias. This variation is most likely to appear in the less readily fermented sugars, especially galactose. With a weakly fermentable sugar, the evolution of gas would be slow, and might not exceed the limit of solubility in the medium. When more cells are present (large inoculum), the saturation point may be exceeded with gas collecting in the fermentation tube.

*We are indebted for this culture to Dr. W. D. Stovall of the Wisconsin State Laboratory of Hygiene, Madison, Wisconsin.

The results of fermentation tests on 7 strains of monilia at the time of isolation and after carrying in stock culture for six to eight months are reported

TABLE II

DIFFERENCES IN FERMENTATION REACTIONS OF MONILIAS DETERMINED BY THREE PERSONS

CULTURE NUMBER	PERSON	DEXTROSE	LEVULOSE	MANNOSE	GALACTOSE	SUCROSE	MALTOSE	LACTOSE	MILK
	A	AG*	AG	AG	A	O	AG	O	C
104	B	AG	A slight	(AG) slight	A	O	AG	O	C
	C	AG	AG	AG	A very slight	O	AG	O	C
	A	AG	AG	A	O	AG	O	O	O
155	B	A slight	O	O	O	O	O	O	O
	C	A	A slight	O	O	O	O	O	C

*A, acid; C, coagulated; G, gas; O, no change.

TABLE III

FERMENTATION REACTIONS OBTAINED WITH SMALL AND LARGE INOCULA FROM AGAR SLANT CULTURES

		AG in Dextrose						
CULTURE NUMBER		LEVULOSE	MANNOSE	GALACTOSE	SUCROSE	MALTOSE	LACTOSE	MILK
155	Small	AG*	AG	A slight	AG	O	O	C
	Large	A	AG	A slight	AG	O	O	C
300	Small	AG	AG	A slight	O	AG	O	C
	Large	AG	AG	AG	A very slight	AG	O	C
303	Small	AG	A (G) slight	A slight	O	AG	O	C
	Large	AG	AG	A	O	AG	O	C
304	Small	AG	AG	AG	O	AG	O	C
	Large	AG	AG	A	O	AG	O	C
305	Small	AG	AG	A very slight	O	AG	O	C
	Large	AG	AG	AG	O	AG	O	C
306	Small	AG	AG	AG	AG	AG	O	O
	Large	AG	AG	AG	AG	AG	O	O
6508	Small	A	AG	A	AG	AG	O	Alk.
0.30	Large	AG	AG	A	AG	AG	O	Alk.
56924	Small	AG	AG	AG	AG	O	AG	C
	Large	AG	AG	AG	AG	O	AG	C

*A, acid; C, coagulated; G, gas; O, no change; Alk., alkaline.

in Table V. Formation of acid in bromeresol purple broth is interpreted by the authors as indicative of production of CO_2. The monilias are not known to produce organic acids as end-products of fermentation. "Acid" then indicates formation of a smaller amount of CO_2, while "acid and gas" indicates the formation of a larger amount. It could not then be said that any change had

occurred in the fermentative characters or sugars fermented by the group of organisms included in this test. An effort was made from the beginning of the work to inoculate fermentation tubes with what appeared to the eye to be similar masses of yeast cells. In spite of this crude method tests were duplicated.

In view of the reputed variability of the monilias, it was of interest to compare the fermentation results published by several workers with the groups proposed by Stovall and Bubolz.[9] Table VI gives this data. Since we have not studied *Monilia parapsilosis*, we have no comments to offer about this species. *Monilia candida*, which ferments sugars more vigorously than *Monilia albicans*, has given no variation in the hands of several workers. The latter has given variable results only on those sugars fermented very slowly, galactose and sucrose. In our own work, acid production has been obtained in sucrose with only a very few strains. Nevertheless, the results, given in Table VI, are surprisingly consistent in view of the many factors which were varied by the several workers. In spite of this agreement, it is not proposed to classify the monilias by means of biochemical criteria alone. However, we do believe that biochemical criteria, in conjunction with morphology, are valuable in the classification of these fungi.

TABLE IV

FERMENTATION REACTIONS OBTAINED WITH SMALL AND LARGE INOCULA FROM GLUCOSE BROTH CULTURES

AG in Dextrose and Levulose

CULTURE NUMBER		MANNOSE	GALACTOSE	SUCROSE	MALTOSE	LACTOSE	MILK
104	Small	AG*	A slight	O	AG	O	C
	Large	AG	A	O	AG	O	C
155	Small	AG	A slight	AG	O	O	C
	Large	AG	AG	AG	O	O	C
300	Small	A very slight	A slight	A very slight	AG	O	C
	Large	AG	A slight	AG	AG	O	C
303	Small	AG	A	O	AG	O	C
	Large	AG	AG	A very slight	AG	O	C
304	Small	AG	AG	A	AG	O	C
	Large	AG	AG	A very slight	AG	O	C
6508	Small	AG	AG	AG	AG	O	Alk.
0.30	Large	AG	AG	AG	AG	O	Alk.
56924	Small	AG	AG	AG	O	AG	C
	Large	AG	AG	AG	O	AG	C

*A, acid; C, coagulated; G, gas; O, not run; Alk., alkaline.

DISCUSSION

It appears from Tables II, III and IV that other factors than the size of the inoculum may be responsible for variation in the fermentation tests on monilias. The data in Tables III and IV were obtained by "C" of Table II. Note the differences between the fermentation reactions of 155 in the three tables. With Strain 300, gas was formed from galactose in the tests given in Table III and not in those of Table IV. Sucrose gave gas in one test and not in

the other. Similar differences are seen in Strains 303 and 304. As yet we have not had the opportunity to investigate other factors which may be operative in giving variable fermentation reactions. A few possibilities are: (a) the age of the inoculum, (b) the interval between previous transfers of the culture, and (c) the composition of the medium in which the inoculum is grown. If these factors were evaluated in relation to fermentation studies on the monilias,

TABLE V

FERMENTATION REACTIONS OF MONILIAS FRESHLY ISOLATED AND AFTER CARRYING IN STOCK CULTURE

AG in Dextrose, Levulose, Mannose, Maltose. No change in Lactose

CULTURE	DATE TESTED	GALACTOSE	SUCROSE	MILK
244	7/25/34	A (G)* very slight	A	C
	3/14/35	A (G) slight	A (G) slight	C
259	7/16/34	A (G) very slight	A	C
	3/14/35	A (G) slight	A (G) slight	C
262	7/31/34	A	A slight	C
	3/14/35	A slight	A very slight	C
264	7/31/34	A	O	C
	3/27/35	A very slight	O	C
265	7/31/34	A	A slight	C
	3/14/35	A slight	A (G) slight	C
268	8/16/34	AG	A slight	C
	3/14/35	A (G) slight	A slight	C
269	8/25/34	AG	AG	O
	3/14/35	AG	AG	O
271	9/14/34	AG	O	C
	3/27/35	A very slight	O	C
272	9/10/34	AG	A slight	C
	3/14/35	A (G) slight	A (G) very slight	C
274	9/17/34	A	O	C
	3/27/35	A slight	O	C
275	9/17/34	A slight	O	C
	3/27/35	A slight	O	C

*A, acid; C, coagulated; G, gas; O, no change.

greater unanimity might be promoted. Only a few cultures show variability; the remaining strains met in our work exhibit remarkably similar reactions representing only a few types of organisms.

In a recent paper Lamb and Lamb[7] proposed that tests for disappearance of sugars be made in addition to the usual observation for production of acid and gas. These authors were dissatisfied with the use of bromeresol purple broth containing Durham tubes because of the reversion from an acid to a more alkaline reaction, occurring at different times in the different sugar broths. This reversion to a more alkaline reaction does not constitute a criticism of the method used. We have made tests which showed that this phenomenon is produced by a loss of CO_2 from the medium near the end of or after the completion

TABLE VI

MONILIAS GROUPED ACCORDING TO STOVALL AND BUBOLZ. FERMENTATION REACTIONS BY VARIOUS WORKERS

	INDICATOR	DEXTROSE	LEVULOSE	MANNOSE	GALACTOSE	SUCROSE	MALTOSE
Monilia parapsilosis:							
Stovall and Bubolz	Bromthymol blue	AG*	AG	AG	A	A	A
Benham	Andrade	AG	AG	A	A	A	Not run
Lamb and Lamb	Andrade	AG	AG	Not run	A	A	O
Monilia albicans:							
Stovall and Bubolz	Bromthymol blue	AG	AG	AG	AG	A	AG
Benham	Andrade	AG	AG	AG	A irreg.	A irreg.	AG
Fineman	Not given	AG	AG	Not run	A	A	AG
Shrewsbury[19]	pH determined	AG	AG	Not run	AG	A	AG
Lamb and Lamb	Andrade	AG	AG	Not run	A	A	AG
Lamb and Lamb (psilosis)	Andrade	AG	AG	Not run	AG	A	AG
Present authors	Bromcresol purple	AG	AG	AG	A or AG	A rarely	AG
Monilia candida:							
Stovall and Bubolz	Bromthymol blue	AG	AG	AG	AG	AG	AG
Benham	Andrade	AG	AG	AG	AG	AG	AG
Shrewsbury	pH determined	AG	AG	Not run	AG	AG	AG
Lamb and Lamb	Andrade	AG	AG	Not run	AG	AG	AG
Present authors	Bromcresol purple	AG	AG	AG	AG	AG	AG

*A, acid; G, gas; O, no change.

of fermentation. This reversion would appear at different times, depending on the rate at which the individual sugars were fermented.

Lamb and Lamb in comparing the disappearance of sugars with the fermentation readings of "acid and gas" demonstrated that sucrose and galactose disappeared from the medium, even though acid was the only indication of fermentation. With this method the less readily fermented sugars would require a longer incubation. In our opinion, the fermentation tests are more practical when a relatively short period is required for their completion. Employing the Durham tube method, and an indicator, as bromeresol purple, these tests can be completed in a week.

The reliability of the fermentation reactions of the monilias has been investigated and at least one cause of variability indicated. If the size of the inoculum is relatively constant, the difficulties encountered in obtaining consistent fermentation reactions may be lessened considerably. This point has been emphasized for the yeasts by Stelling-Dekker[20] and others. The difference between production of acid or acid and gas is only a difference of degree. However, under controlled conditions this difference can be of diagnostic value with but few exceptions. Thus the importance of a standard procedure for such work is further emphasized.

CONCLUSIONS

1. It is proposed that production of acid, or acid and gas, by monilias in sugar broths represents only a difference in degree and that both should be recognized as fermentation.

2. The rates of fermentations of most monilias are sufficiently different to permit separation into groups.

3. In some instances a large inoculum was found to produce fermentation when a small inoculum failed to do so.

4. Eleven strains of monilia had the same fermentation characteristics six to eight months after isolation.

5. A comparison of the fermentation data of several workers for the three types of monilia (Stovall and Bubolz) revealed considerable unanimity.

6. The occasional strains, which give variable fermentations, make morphologic study a desirable supplement.

REFERENCES

1. Castellani, A.: Fungi and Fungous Diseases, American Medical Association, 1928.
2. Ashford, B. K.: The Etiology of Sprue, Am. J. M. Sc. **154**: 157, 1917.
3. Garrod, L. P., and Stone, K.: The Classification of Monilias by Serological Methods, J. Path. & Bact. **34**: 429, 1931.
4. Benham, R. W.: Certain Monilias Parasitic on Man. Their Identification by Morphology and by Agglutination, J. Infect. Dis. **49**: 183, 1931.
5. Benham, R. W.: Monilias, Yeasts, and Cryptococci. Their Pathogenicity, Classification, and Identification, Am. J. Pub. Health **22**: 502, 1932.
6. Wachowiak, M., Marr, J., Hagebusch, O. E., Randall, W. A., and Fleischer, M. S.: Differentiation of Various Strains of Monilia by Cultural Methods, J. Infect. Dis. **54**: 35, 1934.
7. Lamb, J. H., and Lamb, M. L.: A Grouping of the Monilias by Fermentation and Precipitin Reactions, J. Infect. Dis. **56**: 8, 1935.
8. Fineman, B. C.: A Study of the Thrush Parasite, J. Infect. Dis. **28**: 185, 1921.
9. Stovall, W. D., and Bubolz, A.: Forty Strains of Yeast-Like Fungi Isolated From Sputum, J. Infect. Dis. **45**: 463, 1929.

10. Stovall, W. D., and Bubolz, A.: Identification of Certain Funguses Pathogenic for Man, Am. J. Pub. Health **22**: 493, 1932.
11. Stovall, W. D., and Bubolz, A.: Cultural and Biochemical Characteristics of Monilia Isolated From Human Sources, J. Infect. Dis. **50**: 73, 1932.
12. Stovall, W. D., and Bubolz, A. A.: Yeast-Like Fungi, J. Lab. & Clin. Med. **18**: 890, 1933.
13. Stovall, W. D., and Pessin, S. B.: Classification and Pathogenicity of Certain Monilias, Am. J. Clin. Path. **3**: 347, 1933.
14. Hesseltine, H. C.: Diabetic or Mycotic Vulvovaginitis, J. A. M. A. **100**: 177, 1933.
15. Plass, E. D., Hesseltine, H. C., and Borts, I. H.: Monilia Vulvovaginitis, Am. J. Obst. & Gynec. **21**: 320, 1931.
16. Hesseltine, H. C., Borts, I. H., and Plass, E. D.: Pathogenicity of the Monilia (Castellani), Vaginitis and Oral Thrush, Am. J. Obst. & Gynec. **27**: 112, 1934.
17. Hopkins, E. W., and Hesseltine, H. C.: Cultural and Morphological Studies of Cryptococci and Monilias Isolated From Vulvo-Vaginitis and Oral Thrush, J. Lab. & Clin. Med. **21**: 1113, 1936.
18. Fred, E. B., and Waksman, S. A.: Laboratory Manual of General Microbiology, New York, 1928, p. 39.
19. Shrewsbury, J. F. D.: The Genus *Monilia*, J. Path. & Bact. **38**: 313, 1934.
20. Stelling-Dekker, N. M.: Die sporogenen Hefen, Verh. K. Akad. Wetensch. Amsterdam. Afd. Natuurk. 2 Sect. Deel **17**: No. 1, pp. 547.

CULTURAL AND MORPHOLOGIC STUDIES OF CRYPTOCOCCI AND MONILIAS ISOLATED FROM VULVOVAGINITIS AND ORAL THRUSH*

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A GROUP of yeastlike fungi, the monilias and cryptococci, which have been isolated from lesions in various parts of the human body, have received lately increased attention. The interest aroused has led to the proposal of a number of methods for identification and classification.

For a number of years, the system of Castellani,[1] which separated the monilias into species on the basis of the sugar fermentations, was accepted as the only means of identification. Fineman[2] reported that the fermentation reactions of the organisms which she studied were sufficiently constant to be used in identification. The large number of species which resulted from the use of Castellani's system and the difficulty in obtaining similar results on repetition led to numerous criticisms of this method. Later, Castellani[1] came to regard his fermentation method as being of little value unless applied to cultures of recent isolation. Nevertheless, he maintained that certain of the monilias did not change in their action on sugars.

Stovall and Bubolz[3-6] and Stovall and Pessin[7] reopened the question of the use of a biochemical classification of the monilias. A number of cultures which they isolated from various lesions, as well as those obtained from stock collections, were found to be divisible into three groups. Malt agar and milk modified by the addition of calcium lactate were found to be important diagnostic aids in addition to the sugars commonly used. These strains were found to give the same reactions even after prolonged cultivation. No morphologic differences were observed which would allow the separation into species.

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The study of morphology of the monilias has been recommended by a few workers as a means of separating species. Ashford[8] stressed the importance of these observations in addition to the fermentation tests. However, it is difficult to obtain exact morphologic descriptions from his paper. Benham[9, 10] has made recently a distinct contribution in describing the morphology of four common monilia species. The type of growth and giant colonies were also found to possess differential characteristics. Fermentation of sugars was studied but doubt was expressed that sufficient differences were found to be of value, while serologic differences were believed to be the most definite and reliable. Benham and Hopkins[11] have applied the methods of Benham to monilias isolated from the fingers, toes, finger and toe nails, the tongue, and feces of 100 persons, and of the monilia cultures obtained, only 6 were not classifiable into four groups.

Langeron and Talice[12] have recently proposed a morphologic classification of yeastlike fungi. As yet, no papers have been found in which the key of Langeron and Talice has been applied to the monilias.

Serologic classification of the monilias has been advocated by several workers. Benham,[9] and Benham and Hopkins[11] have reported success in distinguishing *Monilia parapsilosis, Monilia albicans, Monilia krusei,* and doubtful success with *Monilia candida* by means of agglutination and agglutinin absorption methods. Garrod and Stone,[13] using complement fixation and precipitin tests, found most of the monilias in their collection to be identical with *Monilia albicans.* Almon and Stovall[14] have reached the conclusion that the monilias cannot be separated by direct agglutination, but that by appropriate agglutinin absorption *Monilia parapsilosis* could be separated from *Monilia albicans* and *Monilia candida. Monilia albicans* and *Monilia candida* were not separable, as no specificity was exhibited toward these strains even by absorbed serums. Lamb and Lamb,[15] reporting on precipitin reactions in the monilias, found 3 serological groups: (a) *Monilia albicans* and *Monilia candida,* (b) *Monilia parapsilosis* and (c) *Monilia krusei.* These workers believed that acid and gas formation in broth and disappearance of sugars could be used for separating the organisms into the same groups.

No attempt is made to review or clarify the confusion existing in the nomenclature which has developed about the pathogenic yeastlike organisms, since Stovall and Bubolz[5] and Benham[9] have adequately reviewed this subject. Until a decision by a special committee is given or the relationship of the yeastlike organisms has been more completely worked out, the name monilia seems to us to be as good as any other.

The yeastlike organisms isolated from vulvovaginitis and oral thrush were run through the fermentation tests and allowed to grow on agar plates for observation of morphology. The groupings determined by these two means are considered here, and the results obtained by the two methods compared.

EXPERIMENTAL DATA

Material from the vulvovaginal infection or oral thrush patches was removed with a swab or sterile instrument and smeared over a Sabouraud's dextrose agar slant. From those slants showing growth of yeastlike cells after one

or more days' incubation at 37° C. material was streaked on Sabouraud's agar plates, and well-isolated colonies picked. The pure culture so obtained was kept in stock culture on Sabouraud's agar.

As soon as possible after isolation, the pure cultures were inoculated from a forty-eight-hour, Sabouraud's agar slant culture into the sugar broths, 2.5 per cent malt broth, milk with additional calcium lactate,[5] dilution plates made with 2.5 per cent malt agar,[5] streaked on carrot plugs, and streaked on Sabouraud's and corn meal agar.

The sugar broths contained 1 per cent of a sugar, 1 per cent peptone, 0.35 per cent beef extract, and 0.5 per cent sodium chloride. Bromcresol purple was the indicator used except when bromthymol blue was tried for comparison. The milk was made up by resuspending 90 gm. of skimmed milk powder in a liter of water and adding bromcresol purple to a concentration of 0.005 per cent.[16] The milk was sterilized by heating for thirty minutes in an Arnold sterilizer on three successive days.

Fermentation tubes were incubated at 37° C. and observed on the second, third, fourth, and seventh days. As has been pointed out in the preceding paper,[17] the production of acid as well as acid and gas by the monilias is interpreted to be due to the formation of CO_2 from dissimilation of sugar, and is called fermentation. An attempt was made to use comparable inocula for the fermentation tests, since the size of inoculum, as indicated previously[17] is an important factor. The estimation of the amount of yeast mass on a loop is a crude measure of the number of cells; however, after a few weeks, little difficulty should be encountered in obtaining consistent fermentations with all but a few cultures.

The organisms were grouped first on ability to produce mycelium on Sabouraud's agar plates. Those organisms failing to produce mycelium were called cryptococcus, and those producing mycelium, monilia. Since none of the cultures formed ascospores on suitable media, no saccharomyces or endomyces were isolated from this group of patients.

RESULTS

The sugars fermented by the cryptococci are given in Table I. Acid and gas were formed in dextrose, lexulose, and mannose by the 8 strains, and acid and gas from maltose by one strain. None of the cultures made any perceptible change in bromcresol purple milk.

The results obtained with the monilias are given in Table II. These fungi are divided into the fermentation groups proposed by Stovall and Bubolz,[3] and the type reactions given by these authors put into the table for comparison with the strains studied. None of the Type 1 monilias were found. Of the Type 2 monilias in 17 of the 67 strains, mycelium was observed in malt broth, but none formed on Sabouraud's agar. This latter type differed slightly in their reactions in sugar broths from those reported by Stovall and Bubolz. All strains produced acid in galactose broth, but formation of gas was variable. Acid in sucrose broth was formed by only a few strains.

Bromcresol purple had been selected as the indicator, because of its lesser sensitivity to slight changes in acidity. In the tests made in parallel with bromcresol purple and bromthymol blue, acid was produced in all cases in sucrose broth with the latter as the indicator, while with the former five strains failed to do so. Since Stovall has made use of the latter indicator, our differences in the production of acid in sucrose broth can be readily explained. However, as Lamb and Lamb have found that sucrose disappeared from broth inoculated with *Monilia albicans* it appears to be advisable for the determination of sugars *attacked but not fermented* to use an indicator changing at a higher pH than does bromcresol purple.

The coagulation of milk modified by the addition of calcium lactate as recommended by Stovall and Bubolz has proved to be a thoroughly reliable test. Strains of Type 2 monilia are occasionally encountered which do not coagulate this milk in three days, but will do so before the seven-day incubation period is completed.

TABLE I

FERMENTATION GROUPS OF THE CRYPTOCOCCI FOUND IN VULVOVAGINITIS

	NUMBER OF STRAINS	DEXTROSE	LEVULOSE	MANNOSE	GALACTOSE	SUCROSE	MALTOSE	LACTOSE	MILK
Group 1	7	AG*	AG	AG	0	0	0	0	0
Group 2	1	AG	AG	AG	0	0	AG	0	0

*A, acid; G, gas; O, no change.

TABLE II

FERMENTATION GROUPS OF THE MONILIAS FOUND IN VULVOVAGINITIS AND ORAL THRUSH IN COMPARISON WITH TYPES OF STOVALL AND BUBOLZ

	NUMBER OF STRAINS	DEXTROSE	LEVULOSE	MANNOSE	GALACTOSE	SUCROSE	MALTOSE	LACTOSE	MILK
Stovall and Bubolz									
Type 1		AG*	AG	AG	A	A	A	0	0
Type 2		AG	AG	AG	AG	A	AG	0	C
Type 3		AG	AG	AG	AG	AG	AG	0	0
Our Strains								*	
Type 1	0								
Type 2	67	AG	AG	AG	A or AG	A rarely AG	AG	0	C
Type 3	2	AG	AG	AG	A slight or AG	AG	AG	0	0
Unclassified									
Type 1	2	AG	AG	AG	A or AG slight	AG slight	AG	0	0
Type 2	2	AGP	AGP	AGP	0	0	0	0	0

*A, acid; C, coagulated; G, gas; P, pellicle.

One of the Type 3 strains produced only slight acidity in galactose broth, while the other strain gave the typical reactions of the Stovall and Bubolz Type 3. These two strains were not producing any symptoms or evidence of pathologic condition. One strain was isolated from the vulva of an apparently normal nonpregnant woman and the other from a stool of a woman without intestinal disturbances. These strains were sufficiently different in both fermentation reactions and morphology to be readily separated from Group 2.

Of the two unclassified groups, the first differed from Stovall's Type 2 only in the production of a slight amount of gas in sucrose broth, but as will be indicated later, this belongs in this Type 2. The second unclassified group appears to be closely related to *Monilia krusei*. It gives the fermentation results reported for that organism by Stovall.

Another criterion used by Stovall for the separation of groups of the monilias is the production of mycelium in forty-eight hours on malt agar. This test was made on about fifty strains. Of the Type 2 monilias tested, in four strains a few colonies showed the presence of some mycelium, and in the remaining strains none was found. The two Type 3 monilias produced abundant mycelium. It was observed, however, that even in strains producing abundant mycelium the formation of mycelium may not be a constant characteristic. It is affected by acidity and by a heavy or light seeding of plates. While this criterion may have some value in the differentiation of monilias when correlated with other tests, our experience has indicated that it should not be emphasized strongly.

An attempt was made to find morphologic groups in the series of cultures examined. The descriptions of Benham for *Monilia albicans* and *Monilia candida* were used as the types for classification into these species. These groups including the description of the types are given. A number of cultures formed mycelium only in malt broth but were not examined for morphology, as all other cultures had been observed on agar. In a few strains, the mycelium developed too poorly on agar to make possible morphologic studies. Eight strains were described insufficiently to make identification possible.

Monilia albicans Type.—Hyphae short to elongate. Branching sometimes frequent. Buds in whorls which later submerge the hyphae, giving the appearance of a string of beads (27 strains).

Subtype of M. albicans.—Like *M. albicans* except that the first buds may elongate, branch, and bear a cluster of conidia at a distance from the hyphae. This form of growth gives the hyphae a ragged appearance. These branches may later embed the hyphae, or the usual balls of conidia may be formed in other parts of the mycelium (4 strains).

Monilia candida Type.—Mycelium very abundant. Buds may at first form in whorls and then other buds appear later between the whorls or the buds may form at irregular intervals from the start. The first buds elongate and branch, giving branched clusters at a distance from the hyphae (2 strains).

Miscellaneous types unlike the above two:

1. Mycelium rather short, little branched. Buds elongate to ovate, placed at irregular intervals along the hyphae. Ragged appearance to old hyphae (2 strains).

2. Dense mycelium with branches of fir tree appearance. Conidia borne on these branches. Dry wrinkled colony growth, pellicle on sugar broths. Probably *Monilia krusei* (2 strains).

3. Mycelium may be much branched, made up of elongated elements constricted at the segments, or may have buds at irregular intervals which elongate and branch (2 strains).

Mycelium not sufficiently developed for observation of morphology (7 strains).

Mycelium formed only in malt broth (17 strains).

Poorly described, morphology uncertain (8 strains).

TABLE III

MORPHOLOGIC GROUPS OF MONILIAS CLASSIFIED BY THE FERMENTATION METHOD OF STOVALL AND BUBOLZ

	NUMBER OF STRAINS
Type 2:	
Monilia albicans	31
Miscellaneous types	4
Mycelium in malt broth only	17
Insufficient development for observation	7
Poorly described	8
Type 3:	
Monilia candida	2

The classifications by fermentation and by morphology were compared to determine the extent of correlation. Stovall and Bubolz Monilia Type 2 is *Monilia albicans* and the Type 3 is *Monilia candida.* Table III gives the results of this comparison. Of the 35 strains classified by fermentation as Type 2 Stovall of which morphologic descriptions were obtained, 31 were *Monilia albicans.* In Table II, the 2 strains in the "Unclassified" fermentation group, Subgroup 1, were found by morphology to be *Monilia albicans.* The Subgroup 2 were considered to be *M. krusei.*

The 2 strains classified by Stovall's method as Type 3 were identified by morphology as *Monilia candida.*

The degree of correlation obtained between fermentation reactions and morphologic observation seems to us to be sufficiently high to warrant further study. If the correlation were firmly established, greater unity could be obtained between the investigations of various workers.

CONCLUSIONS

1. Fermentation groups were determined in 8 cryptococcus and 73 monilia strains isolated from vulvovaginitis of adults and oral thrush of infants.

2. The monilias were readily classified into the types of Stovall and Bubolz, with minor differences which have been indicated.

3. Morphologic observations of Benham were made on the monilias whenever possible, and the cultures separated into species.

4. Of the 35 strains on which morphologic observations could be made and which would be classified biochemically as *Monilia albicans,* 31 were found by morphology to be *Monilia albicans.*

5. For the present it is recommended that both biochemical and morphologic studies be made for the classification of the yeastlike organism.

REFERENCES

1. Castellani, A.: Fungi and Fungous Diseases, American Medical Association, Chicago, 1928.
2. Fineman, B. C.: A Study of the Thrush Parasite, J. Infect. Dis. **28**: 185, 1921.
3. Stovall, W. D., and Bubolz, A.: Forty Strains of Yeast-Like Fungi Isolated From Sputum, J. Infect. Dis. **45**: 463, 1929.

4. Stovall, W. D., and Bubolz, A.: Identification of Certain Funguses Pathogenic for Man, Am. J. Pub. Health **22**: 493, 1932.
5. Stovall, W. D., and Bubolz, A.: Cultural and Biochemical Characteristics of Monilia Isolated From Human Sources, J. Infect. Dis. **50**: 73, 1932.
6. Stovall, W. D., and Bubolz, A.: Yeast-Like Fungi, J. Lab. & Clin. Med. **18**: 890, 1933.
7. Stovall, W. D., and Pessin, S. B.: Classification and Pathogenicity of Certain Monilias, Am. J. Clin. Path. **3**: 347, 1933.
8. Ashford, B. K.: The Etiology of Sprue, Am. J. M. Sc. **154**: 157, 1917.
9. Benham, R. W.: Certain Monilias Parasitic on Man. Their Identification by Morphology and by Agglutination, J. Infect. Dis. **49**: 183, 1931.
10. Benham, R. W.: Monilias, Yeast, and Cryptococci. Their Pathogenicity, Classification and Identification, Am. J. Pub. Health **22**: 502, 1932.
11. Benham, R. W., and Hopkins, A. Mc H.: Yeastlike Fungi Found on the Skin and in the Intestines of Normal Subjects. A Survey of One Hundred Persons, Arch. Dermat. & Syph. **28**: 532, 1933.
12. Langeron, M., and Talice, R.-V.: Nouvelles méthodes d'étude et essai de classification des champignons levuriformes, Ann. de Parasitol. **10**: 1, 1932.
13. Garrod, L. P., and Stone, K.: The Classification of Monilias by Serological Methods, J. Path. & Bact. **34**: 429, 1931.
14. Almon, L., and Stovall, W. D.: Serologic Reactions of Cultures of Monilia and of Some Other Yeastlike Fungi, J. Infect. Dis. **55**: 12, 1934.
15. Lamb, J. H., and Lamb, M. L.: A Grouping of the Monilias by Fermentation and Precipitin Reactions, J. Infect. Dis. **56**: 8, 1935.
16. Fred, E. B., and Waksman, S. A.: Laboratory Manual of General Microbiology, New York, 1928, p. 39.
17. Hopkins, E. W., and Hesseltine, H. C.: Reliability of Fermentation Tests in Identification of the Monilias, J. Lab. & Clin. Med. **21**: 1105, 1936.

FURTHER STUDIES ON COPPER AND IRON IN METABOLISM*

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THE relationship of iron to anemia is a problem that has concerned investigators for a long period of time; it had its origin in connection with studies involving a comparison of the potency of inorganic versus organic iron, which later led to investigations of the relation of solubility of iron compounds to the treatment of anemia. Later still this problem developed into a study of ferric as compared to ferrous compounds in iron therapy and, in the past few years, to the rôle of copper in iron metabolism. Hart, Steenbock, Waddell and Elvehjem[1] were the first to demonstrate that pure iron salts will not cure nutritional anemia; but that the addition of a small amount of copper salt to the iron salt results in hemoglobin synthesis. Keil and Nelson,[2] employing extreme precaution, have arrived at the same conclusion, namely, that copper plays a specific rôle in hemoglobin building. Keil and Nelson[3] not only believe that copper is specific in this respect, but have shown in a previous publication in this Journal that colloidal, as well as crystalloidal, compounds of copper and iron are effective in hematopoiesis. The work performed in the past few years on the rôle of iron and copper in nutritional anemia has further modified our conception of the dietary properties of milk, particularly as regards its copper content.

Investigations in the field of nutrition have shown that cow's whole milk is inadequate for the normal maintenance of albino rats; additions of copper and

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iron to an exclusive milk diet protect against nutritional anemia and produce an apparent well-being in animals of the first generation, as judged by hemoglobin formation, growth, and reproduction. Underhill, Orten, Mugrage, and Lewis[4] have observed no abnormalities in the organs of rats fed milk, copper, and iron for periods of more than a year; Waddell[5] reported sterility in males of the first generation receiving a milk, copper, and iron diet. The sterility was marked by a progressive testicular degeneration coupled with an absence of motile sperm in the seminal fluid. Ferric chloride was supposedly the causative agent. He found marked degeneration of the testes of male rats as early as ten weeks on a milk, copper, and iron diet. Glanzmann[6] observed that rats fed for a year on an exclusive milk diet grew well at first, but later growth slackened and became stationary. Atrophy of testes and mammary glands, coupled with a failure of reproduction and lactation, were noted. Cardiac hypertrophy also resulted. Reproduction and lactation were restored by manganese. Keil, Keil, and Nelson[7] found that the animals on a milk, copper, and iron ration grow more slowly than those on a good stock diet. Reproduction occurred in animals of the first generation, but the second generation did not reproduce. This experience with second-generation rats, together with the findings of Waddell, led us to investigate both males and females of the first and second generations, in order to observe whether or not the apparent sterility on this diet could be due to a degeneration of sex organs. Metabolism studies on females receiving milk, copper, and iron were performed in an effort to bring out any indications of an organic failure when milk, copper, and iron constituted the diet.

EXPERIMENTAL

The results reported in this paper were obtained on rats. All animals were fed whole milk obtained from Holstein cows in the dairy herd of Iowa State College. The cows were fed a good dairy ration designed for maximum milk production and were milked by hand directly into glass jugs. This procedure offered a uniform milk supply and eliminated contamination by foreign matter. Purified copper as $CuSO_4.5H_2O$ and iron as $FeCl_3.6H_2O$ were added to the fresh milk each morning; the salts were made in the laboratory from the purified elements. Spectrographic examinations of the metals were made, in order to insure a high degree of purity. Hemoglobin was determined by the Newcomer method. The animals were bled by the tail.

The males in Table I were killed and the trimmed testes were weighed and placed in Bouin's fixing solution prior to histologic sectioning; the epididymis was cut and semen squeezed into a drop of Ringer's solution on a microscope slide which was immediately examined microscopically for motile sperm. Those animals on the longer experiments were first subjected to mating tests with unbred females from the stock colony. Many females of the second generation on a milk, copper, and iron diet were tested for permanent sterility at the end of an experiment by placing them with mature males reared on a stock diet. Other females were likewise tested but with this difference; all of the rats were fed stock ration during the mating test. The animals described in Table II were taken directly from the milk, copper, and iron diet, killed, and their ovaries

trimmed and weighed. All ovaries were fixed in Bouin's solution for histologic examination. The usual histologic technic for paraffin embedding was followed in the treatment of testes and ovaries. Sections 10 mμ in thickness were stained with Delafield's hematoxylin and examined microscopically; some of the sections were counterstained with eosin, in an attempt to secure better differentiation.

TABLE I

WEIGHTS OF TESTES OF RATS ON DIFFERENT DIETS

FIRST GENERATION					SECOND GENERATION				
WK. ON RATION	BODY WT.	WEIGHT OF TESTES	DONALDSON VALUES	AMT. CU AND FE FED DAILY	WK. ON RATION	BODY WT.	WEIGHT OF TESTES	DONALDSON VALUES	AMT. CU AND FE FED DAILY
30	264	2.48	2.52		32	254	2.66	2.48	
30	190	2.43	2.11		28	203	1.96	2.20	
42	290	1.43	2.65		28	210	2.10	2.24	
42	260	2.93	2.50		28	279	2.56	2.58	0.50 mg. Fe +
24	225	2.45	2.33		28	330	2.76	2.79	0.05 mg. Cu
44	323	1.53	2.78	0.50 mg. Fe +	40	205	2.12	2.20	
44	385	1.81	3.00	0.05 mg. Cu	40	214	2.72	2.67	
40	262	2.49	2.52		36	251	2.56	2.46	
36	234	2.39	2.37		44	223	2.41	2.31	
36	175	1.35	2.00		44	245	2.34	2.44	
36	300	2.94	2.69		40	213	2.32	2.24	5.0 mg. Fe +
40	165	2.40	1.94		40	231	2.46	2.35	0.50 mg. Cu
40	172	2.26	1.98		40	188	2.54	2.09	
48	208	2.12	2.22		Ave.	234	2.42	2.38	
20	213	2.31	2.24		40	180	2.30	2.05	
20	161	2.25	1.89		40	277	2.45	2.58	
20	131	1.47	1.63	1.0 mg. Fe + 0.05	40	255	2.27	2.48	Stock diet
14	211	2.31	2.24	mg. Cu	40	198	1.86	2.15	
14	163	2.25	1.92		Ave.	227	2.22	2.31	
14	135	1.48	1.67						
Ave.	223	2.15	2.26						

TABLE II

WEIGHTS OF OVARIES OF RATS ON DIFFERENT DIETS

FIRST GENERATION					SECOND GENERATION				
WK. ON RATION	BODY WT.	WEIGHT OF OVARIES	DONALDSON VALUES	AMT. CU AND FE FED DAILY	WK. ON RATION	BODY WT.	WEIGHT OF OVARIES	DONALDSON VALUES	AMT. CU AND FE FED DAILY
30	169	0.048	0.0479		36	175	0.036	0.0482	
30	168	0.050	0.0479		36	180	0.046	0.0484	
30	160	0.036	0.0474		36	164	0.032	0.0476	
30	155	0.028	0.0471		36	180	0.040	0.0484	
30	175	0.044	0.0482		36	178	0.030	0.0483	
30	171	0.043	0.0479	0.50 mg. Fe +	36	186	0.036	0.0485	
30	150	0.027	0.0468	0.05 mg. Cu	36	194	0.046	0.0489	
30	179	0.040	0.0483		36	165	0.031	0.0477	
36	187	0.034	0.0487		30	184	0.044	0.0485	0.50 mg. Fe +
36	183	0.036	0.0484		30	158	0.034	0.0473	0.05 mg. Cu
36	185	0.040	0.0485		30	183	0.050	0.0484	
Ave.	171	0.0387	0.0479		30	170	0.044	0.0479	
28	170	0.044	0.0479		30	148	0.036	0.0466	
28	161	0.040	0.0474		30	154	0.035	0.0471	
28	175	0.042	0.0482	Stock diet	30	170	0.044	0.0479	
30	187	0.046	0.0487		30	162	0.042	0.0474	
Ave.	173	0.043	0.0480		30	155	0.046	0.0471	
					Ave.	171	0.0395	0.0479	

Galvanized iron wire cages resting in glass funnels were used for metabolism cages. An iron wire screen in the funnel stem served to catch the feces and allowed the urine to flow into a receiving flask. The urine was collected under a layer of toluene. Six female rats six months of age were employed in each experiment. Metabolism tests were carried out on three groups of animals. One group had been fed milk, copper, and iron from weaning time; another group was depleted of its copper and iron stores by an exclusive milk diet until a state of anemia existed; and a third group was given the regular stock colony ration. Urine samples were taken every six hours and analyzed by recognized methods given in the tenth edition of *Practical Physiological Chemistry* by Hawk and Bergeim.[8]

DISCUSSION

In sterility of male rats caused by a degeneration of the testes, the first symptom noted is a loss in weight of those organs; the testes become soft and a marked edema exists. Spermatozoa are either absent in the epididymis or assume deformities. Since individual variation exists in the weights of normal male testes, it becomes necessary to make histologic studies to determine whether or not the germinal epithelium is intact.

The figures in Table I show weights of testes from animals, both of the first and second generation; these rats were fed upon a milk diet with various amounts of copper and iron supplements. Males of the second generation were reared from parents receiving 0.50 mg. Fe as ferric chloride along with 0.05 mg. Cu as copper sulphate in the milk daily; the figures representing testicular weights taken from Donaldson's[9] averages for normal albino rats of the same body weights are given as a means of comparison. It will be noted that the averages from Donaldson appear to be in good agreement with both the males on a milk, copper, and iron diet and those receiving the stock ration. Four males of the first generation on another experiment were given 1.0 mg of unpurified iron as ferric chloride in the milk daily for twelve months; these animals were started on the ration at thirty days of age and were mated with unbred females at the end of a year on the diet. Normal litters resulted in every case within six weeks after the animals were placed together. Likewise, males of the second generation were tested with similar results.

In the work of Waddell[5] male sterility and testicular degeneration were traced to the daily administration of 0.50 mg. of Fe as ferric chloride; histologic pictures showed degeneration to have occurred in these animals. Some of the rats gave evidence of sterility after ten weeks on a diet consisting of whole milk supplemented with copper and iron. The weights of testes shown in Table I do not point to degenerative changes. Motile sperm were found in the semen of all males, and the histologic picture was normal when the iron dosage was ten times the amount reported to cause sterility. We can offer no direct explanation of the discrepancy between our data and that of Waddell's unless, perhaps, some factor contained in the milk from the two sources was responsible. The milk used in our experiments came from Holstein cows fed a first-class dairy ration, while that used by Waddell was obtained from cows receiving straw as roughage. However, the latter investigator found no deficiency of vitamin E in his ration as judged by female reproductive performance.

Table II offers a comparison of the weights of paired ovaries from females on a milk, copper, and iron diet with those of animals fed an adequate stock ration. Donaldson's values serve as a standard value for albino rats of the same body weights. A review of the data shows our normal females on a stock diet to have ovarian weights slightly below those given by Donaldson but higher than rats from the milk, copper, and iron ration; histologic slides made from the latter group show a normal condition. Further proof that the ovaries are intact in the second generation was obtained by mating tests; second-generation females which did not reproduce on a milk, copper, and iron ration were changed to a stock diet, and normal litters were then produced. This fact would indicate a dietary deficiency rather than the irreversible sterility, which might be expected in ovarian degeneration.

Analyses of the urine from three lots of animals represented in Table III show some differences in the amount of nitrogen bodies excreted. Ammonia nitrogen and creatine N are high in the urines of animals on milk, copper, and iron as compared to the values obtained from animals on the stock ration or milk alone. Creatinine nitrogen is also higher in urine from animals on milk plus copper and iron than in urines from animals on milk alone. When copper and iron are excluded the animals do not grow as rapidly; this failure is probably due to the development of nutritional anemia.

SUMMARY AND CONCLUSIONS

1. No sterility or degeneration of the testes was observed in male albino rats maintained on a diet consisting of a good quality milk, supplemented with various amounts of purified iron as $FeCl_3$ together with copper as $CuSO_4$. The testicular weights from both first- and second-generation males on the above ration compared favorably with those obtained on a stock diet; motile sperm were found in all cases and mating tests were positive.

2. The average weights of paired ovaries from female rats reared upon a diet consisting of good quality milk, supplemented with pure iron as $FeCl_3$ along with copper as $CuSO_4$, is slightly lower than that of controls on a stock diet. This apparent abnormality did not denote sterility, since normal litters have been obtained in the second generation after the rats were given a stock ration in lieu of the milk, copper, and iron diet.

TABLE III

ANALYSIS OF URINE

	NITROGEN IN MG. PER RAT IN 24 HR.			PER CENT OF TOTAL NITROGEN		
	STOCK RATION	MILK	MILK + CU + FE	STOCK RATION	MILK	MILK + CU + FE
Volume	26.8 c.c.	27.0 c.c.	25.5 c.c.			
Spec. gravity	1.012	1.012	1.012			
Total N	183.2	190.8	203.5	100.0	100.0	100.0
Urea N	150.96	163.1	158.13	82.4	85.8	77.7
Ammonia N	13.19	12.67	31.35	7.2	6.66	15.4
Uric acid N	0.732	0.426	0.453	0.400	0.224	0.222
Creatine N	0.423	0.69	1.005	0.231	0.362	0.493
Creatinine N	3.44	1.52	2.15	1.88	0.80	1.06

3. A nitrogen partition in the urine of three groups of female rats fed stock ration, milk, and milk plus copper and iron respectively, shows changes in the excretion of ammonia, creatine, and creatinine nitrogen.

REFERENCES

1. Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A.: Iron in Nutrition. VII. Copper as a Supplement to Iron for Hemoglobin Building in the Rat, J. Biol. Chem. **77**: 797, 1928.
2. Keil, H. L., and Nelson, V. E.: The Rôle of Copper in Hemoglobin Regeneration and in Reproduction, J. Biol. Chem. **93**: 49, 1931.
3. Keil, H. L., and Nelson, V. E.: The Effect of Various Colloidal and Crystalloidal Compounds in Nutritional Anemia of the Rat, J. LAB. & CLIN. MED. **19**: 1083, 1934.
4. Underhill, F. A., Orten, J. M., Mugrage, E. R., and Lewis, R. C.: The Effect of the Prolonged Feeding of a Milk-Iron-Copper Diet to Rats, J. Biol. Chem. **99**: 469, 1932-33.
5. Waddell, J.: Male Sterility on Milk Diets, J. Nutr. **4**: 67, 1931.
6. Glanzmann, E.: Experimentelle Untersuchungen über die Nahrungsdefekte der Kuhmilch und verschiedener Milchpräparate, Ztschr. f. Vitaminforsch. **3**: 2, 1934.
7. Keil, H. L., Keil, H. H., and Nelson, V. E.: The Effect of Addition of Minerals and Sucrose to Milk as Shown by Growth, Fertility and Lactation of the Rat, Am. J. Physiol. **108**: 215, 1934.
8. Hawk and Bergeim: Practical Physiological Chemistry, ed. 10, Philadelphia, 1931, P. Blakiston's Son & Co.
9. Donaldson, H. H.: The Rat, ed. 2, Philadelphia, 1924, Wistar Institute of Anatomy and Biology.

THE VALUE OF THE BIOPSY*

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HISTORICAL

THE word biopsy comes from the Greek words meaning life and vision. At the present time the term is confined to examination of tissue removed from the living body in contrast to necropsy, meaning the examination of tissue from the dead body. Muller in 1838 showed tumors to be composed of cells. Kiwisch (1847), Schuh (1851), and Thiersch (1865) employed the microscope to some extent in surgery. Virchow in 1854 demonstrated that the biopsy was of value but later was led to believe that the study of gross pathology was much more reliable in surgical pathology. His influence was so great at that time that progress in the histologic study of tumors was greatly retarded. It was Ruge (1879), however, who first adopted the microscopic examination of all surgical material, and it is he who is usually referred to as the "Father of the biopsy." He urged the careful microscopic study of all uterine curettings and also bits of the cervix uteri in all doubtful cases. His ideas were opposed by the medical profession of Germany probably due to the powerful influence of Virchow, and it was not until 1889 that the German Surgical Congress finally accepted his teachings. Since that time the biopsy has gradually become almost universally accepted, though there are still those who object to its use or use it too infrequently.

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OBJECTIONS

As pointed out by McGraw and Hartman,[8] the objections to the biopsy may be roughly divided into two classes: (1) That the incision into the malignant neoplasm stimulates local growth and (2) that the trauma incident to incision stimulates dissemination and increases the incidence of distant metastasis. The experimental work of Knox,[27] Tyzzer,[23] Lubarsch,[24] and Wood[26] has done much to discredit these objections. Lubarsch traumatized implanted areas of mouse sarcoma following which he could find no evidence of increased metastasis. Wood, incising into Flexner rat carcinomas found no increase in metastasis. On the other hand, Tyzzer and Knox demonstrated that massage of experimental mouse or rat tumors did cause a dissemination of tumor tissue into the lungs.

In spite of this experimental work there are some who believe the biopsy a dangerous procedure and, in certain instances which will be discussed more fully later, the biopsy is a procedure which should be done only after every other possible clinical and laboratory study has failed to make the diagnosis. Authorities differ widely as to the indications and contraindications of the biopsy. Ewing[30] regarded curettage as a dangerous procedure in cases of carcinoma of the fundus uteri. Bloodgood denied any danger associated with biopsies of the cervix or corpus uteri. Most authorities now regard diagnostic curettage and cervical biopsies as being indicated in any questionable case. Bloodgood[14] in a long series of cases could find no evidence of increased metastasis, or decrease in five-year cures in those breast tumors which had been biopsied.

TECHNICAL CONSIDERATIONS

Welch is given credit for having made the first frozen section in 1891, though Wilson, McCarty, and Bloodgood have been largely responsible for its development since that time. There is a wide variance of opinion as to the efficacy of the frozen section, even at the present time. Warthin[15] as late as 1931 regarded the frozen section as a passing fad and believed paraffin sections much more reliable. At that time he described a twenty-four-hour technic, which is now being used in the Pathology Department at the University of Michigan. Others (McCarty and Broders) place just as much confidence in the rapid frozen section as they do in paraffin sections, and even go so far as to state that in some instances the preparations are more reliable. This, they say, is particularly true in case of unfixed tissue where they believe the cell detail is less distorted by artifacts. In any event, the technic used in the preparation of tissues for diagnosis should be chosen to best fit the training of the pathologist. If the frozen section is used it should in every instance be followed by a paraffin section.

Close cooperation between the surgeon and pathologist is essential for accurate tissue diagnosis. The pathologist must be able to orient the tissue which is sent to him for examination. The presence of the pathologist at the operating table at the time of the biopsy is the ideal way to accomplish this end. Too often the surgeon sends a very small bit of tissue to the laboratory when a more generous piece could as well be had. This, in many instances, makes diagnosis impossible. Rather than to feel it a duty to render an opinion in such a case, it is much safer for all concerned to insist on more material or decline to give an opinion.

SPECIAL REGIONS

No hard and fast rules can be laid down as regards indications and contraindications to the biopsy, but each case and each region under consideration should constitute a separate problem. Again various authorities with wide experience differ markedly in their methods of handling tumors of various organs. Each of the tissues from which neoplasms commonly arise will be briefly considered.

Perhaps the most common region from which biopsies are taken in a general surgical service is the female breast. In many instances, a surgeon of experience does not require a tissue diagnosis in cases of carcinoma of the breast, because the gross pathology is so characteristic. However, there are pathologic entities such as plasma cell mastitis, which so closely resemble acute carcinoma of the breast that a microscopic diagnosis is essential. Fat necrosis is also often misleading so far as the gross picture is concerned. If the tumor is to be examined histologically the entire mass should be removed in order that the pathologist may select the most questionable area for microscopic study. If the frozen section is used it should be done immediately and a report made to the surgeon. If the paraffin method is used, the wound may be sutured and the breast amputated if necessary at a later date. Bloodgood considers two weeks a safe interval between biopsy and radical surgery. It is no longer considered good surgery to remove a portion of a breast tumor for microscopic examination. The reason for this is not the effect of incision on the tumor but because such a small bit of tumor is not a true index of the character of the tumor as a whole.

Interpretation of histologic changes occurring in lymphoid tissue taxes the powers of the most experienced pathologist. The dividing line between inflammatory lesions and early changes seen in some of the lymphoblastoma group is extremely difficult to define. This tissue should always be fixed in Zenker-Formol solution as well as formalin and, unless the diagnosis is evident in the hematoxylin and eosin stain, one of the blood stains such as Dominici or Giemsa should be done. I have found histologic diagnosis on sections made by the frozen method to be extremely unreliable.

With the development of the transurethral resectoscope came a marked increase in the amount of prostatic tissue available for microscopic study. Clinically unsuspected carcinoma of the prostate is not infrequently discovered as a result of routine examination of this tissue. As pointed out by Munger and Angle,[32] the percentage of carcinoma of the prostate has increased since the use of the transurethral resectoscope, their precentage being 13.5 of all cases.

The purpose of microscopic study of tissue removed from the stomach is usually to prove the presence or absence of malignancy. In questionable cases the use of the frozen section is very valuable here, as it is important for the surgeon to know whether or not malignancy exists before proceeding. The choice between gastric resection and simple excision or gastroenterostomy is often made as the result of the pathologist's report. In the face of a negative report, serial sections of a callous ulcer of the stomach may later show carcinoma microscopically in the paraffin sections.

Care should be taken in taking biopsies of the rectum. It is quite essential that the block be taken perpendicular to the mucosa. There are no contraindications to taking biopsies in this region.

Biopsy is the only means of absolute diagnosis of carcinoma of the esophagus. This should be done in each case in which there is a suspicion of malignancy, in view of the fact that the roentgen signs are far from being 100 per cent accurate. Esophageal ulcer and cardiospasm often produce x-ray shadows which are many times wrongly diagnosed carcinoma by competent roentgenologists.

Biopsies of tumors occurring in the main bronchi and trachea should be done whenever the x-ray film of the chest shows pathology located at a point where it is accessible to the bronchoscope. It is of value both from a diagnostic and prognostic standpoint. In those cases where the x-ray shows a questionable neoplasm near the pleura aspiration biopsy may be done.

Histologic examination of neoplasms of the skin is particularly important. Whether or not the entire lesion is excised for study depends somewhat on its character and location. If on the face, and appearing to be a basal cell carcinoma either a portion or all of it may be removed, preferably the entire tumor if it is small. If the lesion shows evidence of belonging to the melanoma group, it should be widely excised and the entire specimen examined histologically. While it is impractical to remove every nevus encountered, it is usually thought best to examine in this manner those exposed to much irritation. In cases of diffuse neurofibromatosis, or Von Recklinghausen's disease, one should remove tumor tissue with a great deal of hesitancy as surgery is followed by malignant transformation in a rather large number of instances.

Small tumors occurring in the mouth should always be removed and examined histologically as many of them show malignant change at an early stage. If the tumor is large, a portion of it should be removed for section.

Microscopic study of lesions occurring on the cervix uteri and in the endometrium is always indicated where there is any suspicion of malignancy. It was these tissues which were first studied by Ruge. In the case of biopsies of the cervix it is essential that the block be removed so as to represent a piece of tissue perpendicular to the mucosa. A small bit of tissue carelessly removed often shows many bizzare and misleading microscopic changes. There are many pitfalls in the microscopic diagnosis of early squamous cell carcinoma of the cervix, even when great care is taken by the surgeon in the removal of the block. Curettage is now one of our most commonly used diagnostic procedures. There is no evidence at the present time that diagnostic curettage is contraindicated in cases of carcinoma of the uterine fundus. It is the only known method to make an accurate diagnosis.

Bloodgood[14] pointed out that biopsy in bone tumors should be done only after every other possible diagnostic means has been exhausted, such as x-ray, Wassermann, response to radiation, etc. Rapid plunging into the biopsy before careful clinical study of the case should be unreservedly condemned. The diagnosis of some bone tumors, such as Ewing tumor, multiple myeloma, and some cases of osteogenic sarcoma, when based on histology alone, is extremely difficult. A pathologic diagnosis based on the histology plus the objective and subjective

clinical findings is much more reliable. Response to radiation as a diagnostic test is very valuable and is indicated particularly where such radiosensitive bone neoplasms, as Ewing tumor or multiple myeloma, are suspected.

CLINICAL VALUE

Biopsy as a diagnostic aid in other than neoplastic diseases is of distinct use in selected cases. Special instruments such as that proposed by Randall[1] have been advocated in cases of endometrial biopsies in which instance it is unnecessary to give any anesthetic. Such a common diagnosis as hyperplastic endometrium, which has recently been shown to be the result of excessive estrogenic hormone, may thus be made with very little inconvenience to the patient.

The study of bone marrow microscopically has been used particularly during the past few years. As in the case of lymph nodes, the most valuable preparations are obtained if special technic is used, including Zenker fixation and staining with Giemsa's stain. Touch preparations such as advocated by Downey stained with double strength Giemsa make beautiful preparations and may, in certain instances be the sole means of making a diagnosis of such difficult entities as the so-called aleucemic leucemia and other blood dyscrasias. This is not a formidable procedure and can be done without causing the patient any great inconvenience. A button is removed from the sternum, touch preparations being made immediately from the undersurface. If there is sufficient tissue, paraffin sections can also be made from the bone marrow. This procedure is of value not only from the standpoint of leucemia but also in obscure cases of anemia.

Small bits of tissue removed from the liver for microscopic examination frequently bring to light the true nature of some obscure cases of intrahepatic pathology. Such an examination is of particular value in such cases as hemochromatosis, early cirrhosis, etc. Graham[31] took biopsies of the liver in patients operated upon for gallbladder disease, in order to determine the histologic changes in cases of chronic cholecystitis.

The splenic puncture is a procedure which is of distinct value in selected cases where the underlying pathology is not readily detected in the clinical study. Such diseases or syndromes, as Gaucher's disease, Banti's disease and aleucemic leucemia, may be thus diagnosed where all other methods fail.

Biopsies of skeletal muscle have been used recently in the detection of arteriolosclerosis in cases of hypertension and in suspected trichinosis.

Besides its diagnostic value, the biopsy may furnish other important information. The degree of differentiation of malignant tumors determined histologically is very valuable as regards prognosis. Certain histologic criteria may predict the radiosensitivity. Microscopic study of metastatic tumor may give a clue as to the point of origin. There is marked difference of opinion as regards the value of grading of tumors as proposed by Broders of the Mayo Clinic. Some believe that it has prognostic significance.

Minute, clinically unsuspected areas of malignancy will be discovered only if all surgical tissue be examined in the pathology laboratory. Such lesions as carcinoid appendix, gastrointestinal tuberculosis, and abdominal Hodgkin's disease may be discovered by the routine examination of the appendix. Microscopic findings in the tonsils, characteristic of the prodromal stage of measles,

have been described by Warthin and others. These would not have been discovered had it not been for routine tonsil examination. An occasional clinically unsuspected lymphosarcoma or carcinoma of the tonsil is also disclosed. Many other reasons can be named for routine tissue examination, which is gradually being accepted as a part of every standard hospital.

As pointed out by Zemansky and others the sectioning and histologic examination of pleural and ascitic fluids often reveals tumor cells in cases of occult carcinoma. The technic is simple and each fluid from a case in which there is suspicion of an existing tumor should be so studied.

That the popularity of the biopsy is gradually gaining ground is evidenced by the recent development of special instruments and methods of procuring tissue. Hoffman[9] recently devised a punch for the removal of small bits of tissue from tumors for microscopic diagnosis. Others[3, 4, 17-20] have described their findings and the advantages of obtaining tissue by means of aspirations of suspected tumor material. This method is very simple and can be done in any office without causing the patient any discomfort. It is particularly applicable in cases of deep-seated tumors. The technic consists of plunging a large needle into the suspected tumor mass, after having first made a nick in the skin, and applying suction as the needle is felt to enter the tumor. The contents of the needle are then blown into a test tube of 10 per cent formalin and the tissue run through as a regular section. This method has been used in breast tumors, bone tumors, suspicious prostatic neoplasms, and in some instances in lymph nodes. It has also been used in lung tumors, spleens, and deep-seated muscle and fascia tumors.

It is important for the pathologist to have an accurate knowledge of the clinical data in each case from which tissue is taken for study. Histologic changes must be weighed in the balance, considering such factors as age, sex, length of illness, etc.

CONCLUSIONS

1. The biopsy is now recognized as a standard diagnostic procedure.
2. Close cooperation between the surgeon and pathologist is essential to accurate diagnosis.
3. Routine histologic examination of all surgical tissues is an essential requirement of every standard hospital.
4. The biopsy should in every instance be preceded by a thorough clinical study.
5. Recently developed methods of doing biopsies are reviewed.

REFERENCES

1. Randall, L. M.: Endometrial Biopsy, Proc. Staff Meetings Mayo Clinic **10**: 143, 1935.
2. Budd, S. W.: Internat. J. Med. & Surg. **47**: 490, 1934.
3. Martin, H. E., and Ellis, E. B.: Aspiration Biopsy, Surg. Gynec. Obst. **59**: 578, 1934.
4. Friedman, M.: Clinical Value of Puncture Biopsies, Radiology **23**: 429, 1934.
5. Coisser, R. M.: Consideration of Tissue Diagnosis, M. Ann. Dist. of Columbia.
6. Guequierre, J. P., and Weidman, F. D.: High Frequency Currents in Performing Biopsies, J. A. M. A. **103**: 1693, 1934.
7. Doege, P. F.: Pros and Cons of the Biopsy, Wisconsin M. J. **33**: 184, 1934.
8. McGraw, A. B., and Hartman, F. W.: Present Status of the Biopsy, J. A. M. A. **101**: 1205, 1933.
9. Hoffman, W. J.: Punch Biopsy in Tumor Diagnosis, Surg. Gynec. Obst. **56**: 829, 1933.

10. Roussy, G., and Leroux, R.: Value of the Biopsy for Physician and Surgeon, J. Med. frac. **22**: 88, 1933.
11. Shafiroff, B. G. P.: Biopsy Question, Am. Med. **37**: 605, 1931.
12. Hellwig, C. A.: Biopsy in Tumors, Arch. Path. **13**: 607, 1932.
13. Donaldson, F.: The Practical Application of the Microscope to the Diagnosis of Cancer, Am. J. M. Sc. **25**: 43, 1853.
14. Bloodgood, J. C.: Biopsy in the Treatment of Malignancy, J. Lab. & Clin. Med. **16**: 692, 1931.
15. Warthin, A. S.: The Clinical Laboratory as an Aid to Surgery, J. Lab. & Clin. Med. **16**: 743, 1931.
16. Zemansky, A. P. J.: Examination of Fluids for Tumor Cells, Am. J. Med. Sc. **175**: 489, 1928.
17. Martin, H. E., and Ellis, E. B.: Biopsy by Needle Puncture and Aspiration, Ann. Surg. **92**: 169, 1930.
18. Stewart, F. W.: Diagnosis of Tumors by Aspiration, Am. J. Path. **9**: 801, 1933.
19. Ferguson, R. S.: Prostatic Neoplasms, Their Diagnosis by Needle Puncture and Aspiration, Am. J. Surg. **9**: 507, 1930.
20. Coley, B. L., Sharp, G. S., and Ellis, E. B.: Diagnosis of Bone Tumors by Aspiration, Am. J. Surg. **13**: 215, 1931.
21. Bloodgood, J. C.: Danger of Incomplete Removal of Small and Apparently Innocent Lesions and the Problems of the Biopsy, Surg. Gynec. Obst. **44**: 413, 1927.
22. MacCarty, W. C.: Indications and Rules for Biopsy, Proc. Staff Meetings Mayo Clinic **4**: 61, 1929.
23. Tyzzer, E. E.: Factors in the Production of Metastasis, J. M. Research **28**: 309, 1913.
24. Lubarsch, O.: The Significance of Trauma in the Origin and Growth of Malignant Tumors, Med. Klin. **8**: 1651, 1912.
25. Knox, L. C.: Trauma and Tumors, Arch. Path. **7**: 274, 1929.
26. Wood, F. C.: Diagnostic Incision of Tumors, J. A. M. A. **73**: 764, 1919.
27. Knox, L. C.: Relationship of Massage to Metastasis in Malignant Tumors, Ann. Surg. **75**: 129, 1922.
28. Terry, B. T.: A New and Rapid Method of Examining Tissue Microscopically for Malignancy, J. Lab. & Clin. Med. **13**: 550, 1928.
29. Didgeon, L. S., and Patrick, C. V.: A New Method for Microscopic Diagnosis of Tumor, Brit. J. Surg. **15**: 250, 1927.
30. Ewing, James: The Incision of Tumor for Diagnosis, New York M. J. **102**: 10, 1915.
31. Graham, E. A.: Hepatitis. A Constant Accompaniment of Cholecystitis, Surg. Gynec. Obst. **26**: 521, 1918.
32. Munger, A. D., and Angle, E. E.: Transurethral Resection of the Prostate With Special Reference to the Follow-Up, Nebraska State M. J. **20**: 326, 1935.

DOES CALCIUM NEUTRALIZE THYROXINE?*

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WHILE iodine deficiency is regarded by most goiter students as the essential cause of endemic goiter, the contention that a positive factor is necessary for the development of goiter is more and more recognized.

Recent experimental work of Tanabe,[1] Hellwig,[2] Thompson,[3] and Remington and von Kolnitz[4] have established the fact that an excessive intake of calcium is a powerful goitrogenic agent. By giving a diet rich in calcium and, at the same time, containing different amounts of iodine, I^5 was able to produce in white rats parenchymatous and colloid goiters.

The part that calcium plays in goiter development is not understood. There is the possibility, as Marine[6] believes, that it acts by neutralizing thyroxine, that is, by creating a relative insufficiency of iodine. Zondek and Reiter[7] observed in tadpoles which were kept in a 1:15 million dilution of thyroxine a precipitate metamorphosis as first described by Gudernatsch.[8] By increasing the calcium content of the water, Zondek and Reiter saw the thyroxine effect getting weaker and weaker, until finally, when the 400 c.c. of water contained 0.4 gm. of calcium chloride and 0.02 mg. of thyroxine, the tadpoles developed more slowly and grew faster than the controls. Calcium in high dose, in their experiments, reversed the thyroxine effect and brought about changes as observed after feeding thymus substance.

These results, if confirmed, would indeed offer a simple biochemical explanation of the goitrogenic effect of calcium. Goiters caused by an excessive intake of calcium could then be regarded as work hypertrophy of the thyroid in response to a relative iodine deficiency.

Since the photographs which accompany Zondek and Reiter's article do not show a definite influence of calcium on the thyroxine effect, a repetition of these experiments seemed indicated. Three hundred larvae of a common toad were used in my study on which this paper is based. All tadpoles were obtained from a single pool and were equal in size and stage of development. They had no extremities and the tail was large, fanlike. One hundred tadpoles served as controls and were placed in 3,000 c.c. of city water. The second group of 100 were kept in 3,000 c.c. of water containing 0.2 mg. of thyroxine, and the third lot was placed in the same amount of water with thyroxine, but with an addition of 4 gm. of calcium chloride. The food was the same for all three groups and consisted of ground meat and wheat gluten.

Representative specimens of each group, at the end of the experiment, are shown in Fig. 1. While after five days the controls still had all the charac-

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teristics of tadpoles, the metamorphosis of the animals in the second and third groups was almost completed. It is evident that the thyroxine effect was not altered in the least by the addition of calcium. Also the decrease in size was

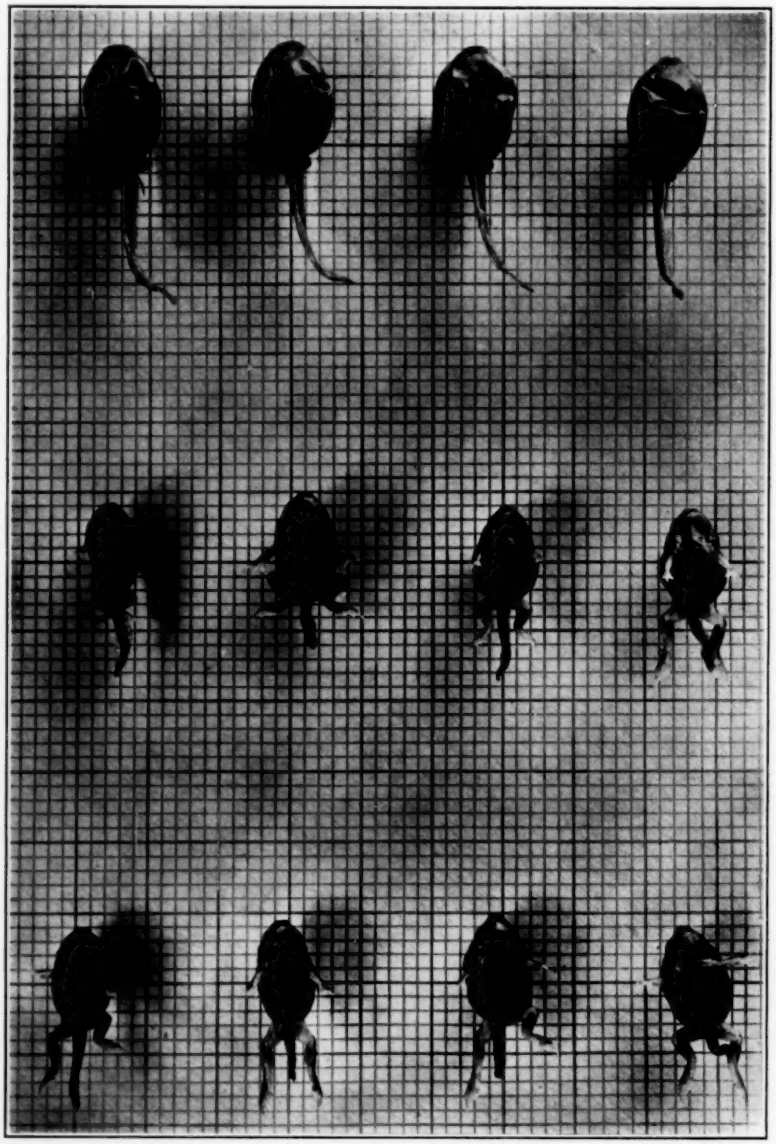

Fig. 1.—Representative specimens of the three experimental groups, killed at the end of the feeding period. First row: Controls. Second row: Thyroxine group. Third row: Thyroxine-calcium group. No difference is noticed between the second and third group.

the same whether calcium was added to the thyroxine or not. The average weight of the controls, at the end of the feeding period, was 800 mg., that of the thyroxine group 343 mg., and that of the thyroxine calcium group 335 mg. Added calcium did not lessen the toxicity of thyroxine. While of the con-

trols only 6 animals died during the five days, 89 tadpoles died of Group 3 which had received thyroxine and calcium compared with 84 of the thyroxine group.

The discrepancy between Zondek-Reiter's and my own results might have been caused by a greater potency of the thyroxine Squibb which I used. In a second experimental series, I gave, therefore, only 0.1 mg. thyroxine, while the dose of calcium added to the water of the third group was the same. There were 15 tadpoles in each of the three groups. Again, after seven days, no influence of calcium on the thyroxine effect could be noticed. Rapidity of metamorphosis, decrease in body weight, and death rate was just as marked in the third group as in the second group which was given thyroxine only.

SUMMARY

The question whether calcium neutralizes thyroxine was studied on tadpoles which are regarded as the most sensitive test animals for the thyroid hormone. Of 300 tadpoles, 100 were placed in a 1:15 million dilution of thyroxine, 100 in thyroxine-water of the same concentration to which calcium chloride was added, while 100 larvae served as controls.

No difference was noticed between tadpoles fed thyroxine alone and those fed thyroxine and calcium, with regard to rapidity of metamorphosis, decrease in body size and death rate. These changes were uniform, even after giving only 1:30 million thyroxine.

The results of my studies do not support the view that calcium neutralizes thyroxine. There is no proof that goiters produced by calcium-rich diets are due essentially to a relative iodine deficiency. It seems more likely that calcium exerts a direct effect on the thyroid gland, causing proliferation of the acinar epithelium.

REFERENCES

1. Tanabe, H.: Experimenteller Beitrag zur Atiologie des Kropfes, Beitr. z. path. Anat. u. z. allg. Path. **73**: 415, 1925.
2. Hellwig, C. A.: Iodine Deficiency and Goiter, Arch. Path. **11**: 709, 1931.
3. Thompson, J.: Influence of the Intake of Calcium on the Thyroid Gland of the Albino Rat, Arch. Path. **16**: 211, 1933.
4. Remington, R. E., and von Kolnitz, H.: Studies on the Relation of Diet to Goiter, J. Nutrition **6**: 325, 1933.
5. Hellwig, C. A.: Experimental Goiter. Functional, Chemical and Histologic Studies, Arch. Path. **19**: 364, 1935.
6. Marine, D.: The Pathogenesis and Prevention of Simple or Endemic Goiter, J. A. M. A. **104**: 2334, 1935.
7. Zondek, H., and Reiter, T.: Hormonwirkung und Kationen, Klin. Wchnschr. **2**: 1344, 1923.
8. Gudernatsch, J. F.: Feeding Experiments on Tadpoles, Am. J. Anat. **15**: 431, 1914.

ADMINISTRATION OF EPINEPHRINE BY INHALATION*

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OF ALL drugs which have been used for the relief of asthmatic paroxysms, epinephrine remains supreme. Its use in this field has been principally confined to administration by hypodermic injection. Recent investigations by the authors,[1] however, have demonstrated the feasibility of obtaining comparable, and in many instances superior, results by inhalation. Success with the latter method is dependent on two factors: first, the use of a stronger epinephrine solution, preferably a 1:100 dilution, and second, an atomizer capable of delivering a fine vapor-like spray.

For nebulization of the 1:100 solution, the majority of the available atomizers were found to be unsuitable, either because of the presence of metal parts

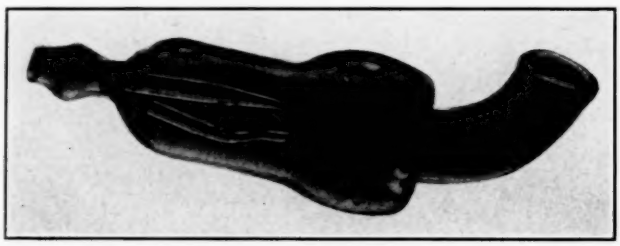

Fig. 1.—Hand atomizer for administration of 1:100 epinephrine solution by inhalation.

which oxidize the epinephrine, or because of certain features of design, or the unevenness of the delivered spray. The instrument, Fig. 1, finally designed was so constructed that satisfactory nebulization could be obtained with but eight or ten drops of solution, a feature desirable in that it conserves the drug by minimizing the amount of the solution exposed to oxidation. Furthermore an inverted moat was incorporated into the instrument to prevent spilling during use, for in the earlier trials several patients accidentally swallowed some of the solution and suffered severe abdominal pain as a result. An ordinary rubber bulb furnishes the air pressure for nebulization.

The method of using the atomizer is quite simple but requires, nevertheless, a modicum of coordination which occasional patients find difficult to acquire. The nozzle of the atomizer is placed just within the open mouth, and the patient inhales deeply while creating a spray. The amount of inhalation necessary for relief varies for each patient and depends not only on the severity of the symptoms, but also on the manner in which the atomizer is manipulated. There is a wide margin of tolerance before any unusual reactions occur. However, until familiar with the procedure, the patient is advised to proceed slowly, allowing one to two minutes to elapse between every five or six deep inhalations.

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For use with children unable to cooperate in the use of the hand atomizer, a special apparatus was devised. This includes first of all, a mask designed to

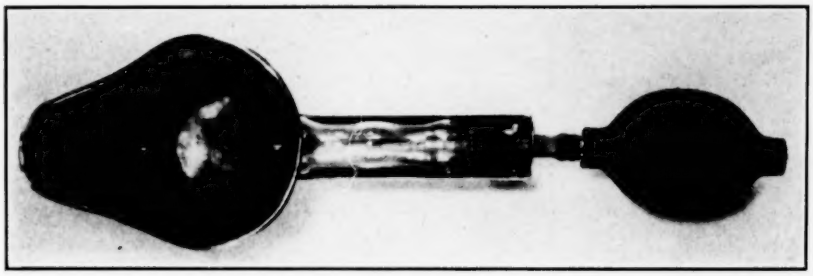

Fig. 2.—Atomizer and metal face mask for inhalation of adrenalin by infants and children.

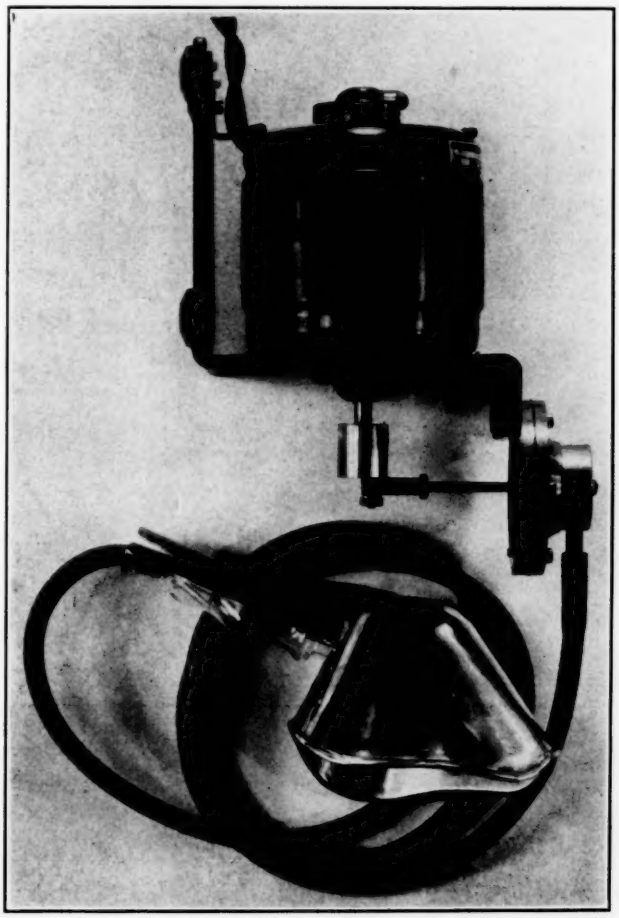

Fig. 3.—The portable air compressor, mask, and atomizer for prolonged administration of adrenalin by inhalation

fit the face, covering the nose and mouth. An atomizer of somewhat different external design fits into the handle with the nozzle projecting into the mask and directed toward its closed end. Air pressure may then be furnished either by

the rubber bulb, Fig. 2, or by a small motor-driven pump, Fig. 3. With either of these instruments, a large volume of vapor may be maintained in the mask while being held over the face of the patient. Its advantages with small children are obvious, and in actual practice, it is an easy matter to administer satisfactorily the solution to small noncooperative patients. The motor apparatus is extremely useful in severe attacks, when medication is needed at frequent intervals. It also fulfills a need in adults when nursing care is not available, and the patient is so weakened by asthmatic distress that the exertion of using the hand bulb is exhausting. The time of exposure with the mask apparatus varies as with the hand instrument, depending principally, however, on the severity of symptoms. Fifteen seconds is the minimum exposure but as much as three minutes have been used in a single exposure, without unusual symptoms of toxicity. In general, less than a minute usually suffices for a single treatment.

The results obtained by the inhalation procedure have been almost uniformly successful. Failure to obtain initial relief is usually due to improper manipulation of the atomizer or to poorly constructed instruments. Three hundred and fifty individuals of all ages, from eleven months to eighty years, have so far been instructed in the use of this inhalation technic. Attacks of all grades of severity have been treated. Severe paroxysms require more prolonged exposure, at times intermittently for a period of fifteen to thirty minutes before relief is obtained. In these instances, however, the decreased tidal air prevents inhalation of an amount of the drug sufficient to give relief in the usual time. The ideal routine in such cases is the administration of hypodermic epinephrine to obtain initial relaxation. This is followed by the use of the inhalation procedure on experiencing the least suggestion of a return of symptoms.

With the average patient, the physiologic effect occurs more rapidly after inhalation of the 1:100 solution than with hypodermic injection; rarely is it any slower. Inhalation is infrequently accompanied by any of the disagreeable side effects of subcutaneously administered epinephrine. So satisfactory have been the results obtained, that the authors feel the inhalation procedure may well supplant in many instances, at least, the use of epinephrine subcutaneously. Furthermore its superiority to the numerous proprietary remedies administered by inhalation should allow the medical profession to combat more adequately the widespread use of these nostrums.

REFERENCE

1. Graeser, J. B., and Rowe, A. H.: Inhalation of Adrenalin for the Relief of Asthma, J. Allergy 6: 415, 1935.

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PRODUCTION OF THE BASOPHILIA OF THE GRANULES OF THE POLYMORPHONUCLEAR NEUTROPHILIC LEUCOCYTES IN EXPERIMENTAL INFECTION OF THE RAT*

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IN CERTAIN infections in man, the granules of the polymorphonuclear neutrophilic leucocytes have a greater affinity for methylene blue than have normal neutrophiles. This basophilia of the granules appears to be a definite indication of infection, but it may be seen in other blood conditions and also follow x-ray therapy. Certain problems relating to the onset of the change of the staining character of the granules, the duration, relation to the types of infecting organism, and the appearance of the phenomenon in the blood of organisms other than man are of interest.

Cesaris-Demel[1] was the first to describe the difference in staining quality and increased granulation of the polymorphonuclear neutrophiles.

Mommsen,[2] Gloor,[3] and others have demonstrated the presence of these deeply staining "toxic" granules in lobar pneumonia. Mommsen used a buffered solution of pH 5.4 with Giemsa's stain to demonstrate the presence of these granules. He termed them "pathologic" or "toxic" granules.

Matis[4] believed Mommsen's granules to be identical with those described by Naegeli as "toxic" or "toxic infectious" granules.

Rosenthal and Sutro[5] noted two kinds of granules, small granules and large granules. They found the small ones in mild infection and during convalescence, and the larger "toxic" granules in severe infection. They believe these pathologic cytoplasmic alterations ("toxic granules") are a valuable aid in the diagnosis and prognosis of pneumonia.

From a study of cytoplasmic changes in the peripheral neutrophiles, Meranze, Mendell, and Meranze[6] found "toxic" changes in pneumonia, peritonitis, bacteriemias, and septicemias, generalized types of infection. In localized infections and noninfectious diseases, the neutrophilic leucocytes did not manifest these changes.

Sutro[7] found the presence of "toxic" granules a constant factor in generalized infection; Spaeth[8] believed that the cytoplasmic changes depend on the virulence of the infecting organism; Fitzhugh[9] used the basophilia of the cytoplasmic granulation of the neutrophilic series of leucocytes as a criterion of the age of the neutrophiles.

The present study was undertaken to produce these cytoplasmic changes in the polymorphonuclear neutrophilic leucocytes of the peripheral blood of the rat. The points considered in the experiment were: When do the changes appear? Do they appear in the older (multilobed) forms or in the younger

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("band forms")? How long do the changes persist? Do all the polymorphonuclear neutrophiles show these changes?

Materials and Methods.—Eighteen young albino rats were used for this experiment. They were given the regular diet consisting of grain, cereals, lettuce, and pantry scraps.

Twenty-four-hour growths of *Staphylococcus aureus* and *Streptococcus hemolyticus* were the infecting organisms. *Staphylococcus aureus* grown on blood agar was used for Group V.* This organism was also used for those experiments in which the effect of killed bacteria was demonstrated. The doses of the organism suspended in physiologic salt solution varied from 0.5 c.c. to 4.0 c.c. The solution contained approximately 150 billion bacteria per cubic centimeter. The injections were given intraperitoneally, except the doses given for reinfection of Group I, which were given subcutaneously.

TABLE I

GROUP I

Aged six weeks
Infecting organism, *Staphylococcus aureus*
Place of injection, intraperitoneal
Place of reinjection, subcutaneous

DAY OF INFECTION	DOSE	PERCENTAGES OF (A) POLYMORPHONUCLEAR LEUCOCYTES, (B) NEUTROPHILES SHOWING BASOPHILIC GRANULES					
		ANIMAL A		ANIMAL B		NORMAL ANIMAL	
		(a)	(b)	(a)	(b)	(a)	(b)
1*	0.5 c.c.‡	16	0	21	0	17.5	0
2		49	0	43	0	20.0	0
3		33	0	25	0	15.0	0
4		22	0	21	0	21.0	0
5		31	0	15	0	19.0	0
6†	1.0 c.c.	30	0	28	0	16.0	0
7		28	0	19	0	22.0	0
8		17	0	21	0	23.0	0

*Day of injection.
†Day of reinjection.
‡1 c.c. contains approximately 150 billion bacteria.

TABLE II

GROUP II

Aged six weeks
Infecting organism, *Streptococcus hemolyticus*
Place of injection, intraperitoneal

DAY OF INFECTION	DOSE	PERCENTAGES OF (A) POLYMORPHONUCLEAR LEUCOCYTES, (B) NEUTROPHILES SHOWING BASOPHILIC GRANULES					
		ANIMAL A		ANIMAL B		NORMAL ANIMAL	
		(a)	(b)	(a)	(b)	(a)	(b)
1*	1.5 c.c.‡	20.5	0	21.0	0	18.5	0
2		37.0	0	48.0	0	22.0	0
3		19.0	0	23.5	0	19.0	0
4		18.0	0	25.0	0	15.0	0
5†	1.5 c.c.‡	23.0	0	17.0	0	22.0	0
6		22.5	0	25.0	0	15.0	0
7		21.0	0	16.0	0	12.0	0

*Day of injection.
†Day of reinjection.
‡1 c.c. contains approximately 150 billion bacteria.

*See data in Tables I to V.

TABLE III

GROUP III

Aged five months
Infecting organism, *Streptococcus hemolyticus*
Place of injection, intraperitoneal

DAY OF INFECTION	DOSE	PERCENTAGES OF (A) POLYMORPHONUCLEAR LEUCOCYTES, (B) NEUTROPHILES SHOWING BASOPHILIC GRANULES					
		ANIMAL A		ANIMAL B		NORMAL ANIMAL	
		(a)	(b)	(a)	(b)	(a)	(b)
1*	2.5 c.c.	17.5	0	22	0	21	0
2		40.0	0	39	0	15	0
3†	2.5 c.c.	22.5	0	26	0	24	0
4		28.0	0	16	0	17	0
5		22.5	0	19	0	13	0
6		17.0	0	22	0	19	0

*Day of injection.

†Day of reinjection.

‡62 per cent consisted of 14 per cent young cells ("band forms") or all the young cells, and 48 per cent of the older (multilobed) forms.

86 per cent consisted of 14 per cent young cells ("band forms") or all the young cells, and 72 per cent of the older (multilobed) forms.

TABLE IV

GROUP IV

Aged five months
Infecting organism, *Staphylococcus aureus*
Place of injection, intraperitoneal

DAY OF INFECTION	DOSE	PERCENTAGES OF (A) POLYMORPHONUCLEAR LEUCOCYTES, (B) NEUTROPHILES SHOWING BASOPHILIC GRANULES					
		ANIMAL A		ANIMAL B		NORMAL ANIMAL	
		(a)	(b)	(a)	(b)	(a)	(b)
1*	2.5 c.c.	17	0	17.0	0	19.0	0
2†	2.5 c.c.	46	0	38.5	0	24.0	0
3†	2.5 c.c.	33	62‡	20.0	14	16.0	0
4		34	86‡	28.0	23	18.5	0
5		32	0	21.0	0	16.0	0
6				24.0	0	12.0	0

The bone marrow of Animal A did not show any basophilic granules on the fourth day of infection.

*Day of injection.

†Day of reinjection.

‡62 per cent consisted of 14 per cent young cells ("band forms") or all the young cells, and 48 per cent of the older (multilobed) forms.

86 per cent consisted of 14 per cent young cells ("band forms") or all the young cells, and 72 per cent of the older (multilobed) forms.

Blood films were made daily. The blood was taken from the tip of the tail, spread on No. 1 cover glasses, and stained with Wright's stain by the following slightly modified method: The concentrated solution was diluted at the end of one minute according to the usual technic but after dilution with water, the mixture was allowed to stand for ten minutes. Brilliant cresyl blue stain was also used to insure a closer observation of changes in staining of the neutrophiles.

Two hundred leucocytes were counted to determine the percentage of polymorphonuclear neutrophilic leucocytes present, and one hundred neutrophiles were counted to obtain the percentage of cells showing changes in staining reaction.

The bone marrow was studied by making impressions of the marrow of the ribs, sternum, and femur on cover glasses by the abklatsch or touch method and

stained with Wright's stain by the usual technic. Serum suspension films were also made and stained in the same way.

TABLE V

GROUP V

Aged twelve weeks
Infecting organism, *Staphylococcus aureus*
Place of injection, intraperitoneal

DAY OF INFECTION	DOSE	PERCENTAGES OF (A) POLYMORPHONUCLEAR LEUCOCYTES, (B) NEUTROPHILES SHOWING BASOPHILIC GRANULES					
		ANIMAL A**		ANIMAL B†		NORMAL ANIMAL	
		(a)	(b)	(a)	(b)	(a)	(b)
1*	4.0 c.c.	11	0	22	0	16.5	0
2*	4.0 c.c.	49	0	36	0	22.0	0
3		40	88‡	31	0	25.0	0
4				27	0	18.0	0

The bone marrow of Animal A showed heavy basophilic granules on the third day of infection.

*Day of injection.

**Animal A was injected with living organisms.

†Animal B was injected with dead organisms.

‡88 per cent consisted of 12 per cent young cells ("band forms") and 76 per cent older (multilobed forms).

The 12 young cells and 52 per cent of the older forms contained many basophilic granules and the remaining 24 per cent contained fewer granules with an affinity for methylene blue.

DISCUSSION

The data show that *Staphylococcus aureus* and *Streptococcus hemolyticus* produce similar results when injected into the rat. With both these organisms, there is an early increase in the number of polymorphonuclear neutrophilic leucocytes, but both fail to maintain this increase for any considerable length of time. A second injection of either streptococcus or staphylococcus given after the animal has recovered from a previous injection of that organism produces only a slight increase in the number of neutrophiles.

In Groups I, II, and III a single injection did not produce an appreciable basophilia of the granules. A second dose given after the effects of the first dose had worn off did not produce basophilia of the granules of the polymorphonuclear neutrophilic leucocytes.

Injections given on successive days with Groups IV and V were followed by staining changes in the polymorphonuclear neutrophiles (basophilia of the granules). With Group IV, however, the basophilia of the neutrophiles was found only in the peripheral blood, while with Group V the change extended to the bone marrow.* This change appeared twenty-four hours after the second successive injection and persisted for forty-eight hours. All the young forms ("band forms") showed the change in the staining but this was not true of all the older (multilobed) forms. At no time during the infection did all the neutrophiles show basophilia of the granules.

There was no basophilia of the granules of the neutrophiles of the blood of the rats injected with dead bacteria.

*It required larger doses and a more virulent organism to produce the change in the bone marrow.

SUMMARY AND CONCLUSION

Rats react similarly to *Staphylococcus aureus* and *Streptococcus hemolyticus* by showing an increase in the number of polymorphonuclear neutrophilic leucocytes. They recover rapidly from infection and show a relative "immunity" to the infecting organism by failure to respond to a second dose after having recovered from a previous dose of the particular organism. It is necessary to give larger doses at short intervals (successive days) to produce basophilia of the granules of the neutrophiles of the peripheral blood. Larger doses of a more virulent organism are necessary to produce staining changes in the bone marrow. All the young polymorphonuclear cells ("staff forms"), but not all the older (multilobed) forms, show basophilia of the granules. Many of the neutrophiles have large "toxic" granules, while the basophilic granules of some of the remaining neutrophiles were scanty and interspersed among the neutrophilic granules.

It may be concluded that basophilia of the granules of the polymorphonuclear neutrophilic leucocytes may be produced experimentally in rats, that the infection must be sustained for at least forty-eight hours to produce this change, that the change occurs in both the younger ("band forms") and the older (multilobed) forms, that basophilia of the granules of the peripheral blood persists for forty-eight hours from the time the change appears, and that larger doses, capable of overcoming the resistance of the rat, are necessary to bring about a greater affinity for methylene blue stain in the younger cells in the bone marrow.

REFERENCES

1. Cesaris-Demel, A.: Ueber die morphologischen Struktur mit den morphologischen und chromatischen Veraenderungen der Leukozyten, Virchows Arch. f. path. Anat. **195**: 1, 1908.
2. Mommsen, H.: Die Pathologische ("toxische") granulation der feingekernten Leukozyten, ihre objektive Erkennung und praktische klinische Verwertung, Klin. Wchnschr. **42**: 2420, 1929.
3. Gloor, W.: Die klinische Bedeutung der Qualitativen Veraenderungen der Leukozyten, Leipzig, Georg Thieme, 1929.
4. Matis, quoted from Schilling, V.: The Blood Picture and Its Clinical Significance, translated by R. B. H. Gradwohl, St. Louis, 1929, The C. V. Mosby Company.
5. Rosenthal, N., and Sutro, C. J.: Blood Picture in Pneumonia, With Special Reference to Pathological Changes in Neutrophils, Am. J. Clin. Path. **3**: 181, 1933.
6. Meranze, D., Mendell, T., and Meranze, T.: Cytoplasmic Changes in the Peripheral Neutrophil as an Aid in Diagnosis and Prognosis, Am. J. M. Sc. **189**: 639, 1935.
7. Sutro, C. J.: Changes in Circulating Leukocytes in Infection, Arch. Int. Med. **51**: 747, 1933.
8. Spaeth, E.: Über die Bedeutung der Granulaveränderungen der feingekörnten Leukocyten, Ztschr. f. klin. Med. **118**: 406, 1931.
9. Fitzhugh, T., Jr.: The Age of the Leukocyte in Relation to Infection, J. LAB. & CLIN. MED. **17**: 975, 1932.

THE BLOOD SUGAR LEVEL AFTER PROLONGED CARBOHYDRATE FEEDING*

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MANY hold that a major contributing cause of human diabetes is an intemperance in carbohydrate consumption.[1-7] In sharp contradistinction with man, idiopathic diabetes in domestic animals is almost unknown, and the only practical means by which experimental diabetes, which can be said to resemble diabetes mellitus, has been induced in the lower forms, involves some form of injury to the pancreas or, more precisely, to the islets.

Several years ago a research involving these and other problems was started in this laboratory upon rats in which the animals were fed the usual stock diet plus 25 per cent sucrose.

This research has progressed now to the point where four generations have been born and raised to maturity upon this diet. There has been no indication of idiopathic diabetes and but one case of marked obesity. A detailed study of the blood sugar characteristics follows.

Twenty-eight rats at the age of 100 days were placed upon the normal diet plus 25 per cent sucrose. Blood sugars were taken at intervals over a period of nineteen months, after which time it was no longer possible to obtain samples by the usual method. From these original 28 rats, four generations of offspring have been and are being followed, each generation being bled as early as size of the animal permitted. Because of other work on hand, it was not always possible to make these tests at any specified age; hence, the variations in the time intervals of the various tables. The Somogyi microblood sugar method[8] has been used throughout the research. Tables I to VI summarize the results so far obtained.

Table I

Original Animals

No. of animals	Time on high sugar diet	Blood sugar level
50	Controls	108 mg.
28	17 days	123 mg.
28	24 days	124 mg.
28	34 days	126 mg.
28	110 days	107 mg.

During the first month on high carbohydrate, the animals showed an elevated blood sugar. After 110 days the blood sugar had reached normal and remained so throughout the 600 days they were observed.

*From the Department of Physiology, College of Physicians and Surgeons, Columbia University in the City of New York.

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Seven animals bled at 94 days of age showed a high sugar level but subsequent observations carried on through 500 days were all normal.

All second generation animals showed normal blood sugar levels at the time of observation and throughout the 575 days observed.

TABLE II

FIRST GENERATION

NO. OF ANIMALS	TIME ON HIGH SUGAR DIET	BLOOD SUGAR LEVEL
50	Controls	108 mg.
7	94 days	122 mg.
23	150 days	102 mg.
41	260 days	100 mg.

TABLE III

SECOND GENERATION

NO. OF ANIMALS	TIME ON HIGH SUGAR DIET	BLOOD SUGAR LEVEL
50	Controls	102 mg.
6	81 days	108 mg.
21	220 days	100 mg.

TABLE IV

THIRD GENERATION

NO. OF ANIMALS	TIME ON HIGH SUGAR DIET	BLOOD SUGAR LEVEL
50	Controls	103 mg.
17	138 days	102 mg.
12	240 days	107 mg.

TABLE V

FOURTH GENERATION

NO. OF ANIMALS	TIME ON HIGH SUGAR DIET	BLOOD SUGAR LEVEL
50	Controls	103 mg.
5	80 days	92 mg.
7	120 days	110 mg.
7	130 days	102 mg.

All third generation animals showed normal blood sugar readings throughout the 500 days they were observed.

The first two readings taken on the fourth generation show a considerable deviation from each other. It is possible that these represent the extreme variations for the animals. The third and last observation made showed normal sugar level.

Two years after this experiment was begun, the question arose as to whether or not the initial rise in the blood sugar level as observed with the original rats could be duplicated. Consequently 12 rats about 90 days of age were placed on the high sugar diet.

TABLE VI

NO. OF ANIMALS	TIME ON HIGH SUGAR DIET	BLOOD SUGAR LEVEL
50	Controls	103 mg.
12	14 days	116 mg.
12	40 days	108 mg.
12	55 days	100 mg.

It will be seen that these rats, as did the original ones, showed an elevated blood sugar, the first few weeks after which time it returned to the normal level.

DISCUSSION

As has been shown, we have no evidence, so far, of any over-fatiguing of sugar tolerance. What is of interest to us is the initially high blood sugar following the administration of a high carbohydrate diet. In both cases, this high level returned to the normal and remained so throughout the periods observed. Just what mechanism is involved in this process of return to normal of the blood sugar level we are at present unable to say. A few of the animals showed some increased weight, but with the exception of one animal, this has not seemed significant. The question of increased liver storage as well as of increased metabolic rate might be considered. This colony is being continued with the hope that light may be thrown upon some, at least, of the various factors involved.

REFERENCES

1. Joslin, E. P.: The Treatemnt of Diabetes, ed. 3, Philadelphia, Lea and Febiger.
2. Joslin, E. P., Dublin, L. I., and Marks, H. P.: Am. J. M. Sc. **186**: 753, 1933.
3. Stone, W. J.: J. A. M. A. **95**: 711.
4. Editorial: J. A. M. A. 202, 1930.
5. Emerson, H.: Cited in Editorial of Medical News **83**: 2099.
6. Nicoll, M., Jr.: From Health talk broadcast, Station WGY, Schenectady, November 15.
7. Wendt, L. F. C., and Peck, F. B.: Am. J. M. Sc. **181**: 52, 1931.
8. Somogyi, M.: J. Biol. Chem. **70**: 599, 1926.

THE RELATION OF THE NONFILAMENT AND FILAMENT COUNTS DURING EXCITEMENT*

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A VARIETY of conditions may cause a deflection of the nonfilament and filament neutrophile counts. Danzer (1930) observed that the destruction and absorption of various tissues in vivo is followed by a deflection of the count to the left. He states, "Extensive destruction of tissues in vivo, from whatever cause, results in the liberation into the blood stream of substances which stimulate the leucopoietic centers." Katz and Nice (1934) in their study of the chemical and cytologic changes in the blood during excited states found a rapid breakdown of tissue products during states of anger, fear, and pain. This was manifested by an increase in the nitrogenous constituents of the blood during these states and was accompanied by emotional leucopenia in the rabbit. Recovery to the normal neutrophile level took place within fifty or sixty minutes after the excited period.

In this investigation, we have studied the effect of the products of tissue breakdown during excitement upon the bone marrow activity as shown by the number of new neutrophiles set free into the blood stream, the cause of the emotional leucopenia in our rabbits, and the effect of excitement upon the relation of the number of the nonfilament to the filament cells.

Procedure.—Healthy adult rabbits which were accustomed to being handled were used in these experiments. A drop of blood was obtained by puncturing an ear vein and cover slip smears were made and stained with Wright's stain.

The normal nonfilament and filament counts were obtained at two consecutive thirty-minute intervals, after which the animal was excited by the method previously described from this laboratory. Blood smears were made immediately following the excitement and thirty and sixty minutes, respectively, after this period. One hundred neutrophiles were enumerated in each count.

We have followed the Farley, St. Clair, and Reisinger (1930) modification of the Cooke and Ponder method in making these counts. In this modification the neutrophiles are divided into two groups: nonfilament (young cells) and filament (old cells). In the young neutrophiles, the nucleus is unsegmented, while in the filament group, fine threads of chromatin material connect two or more segments of the nucleus. We adhered closely to the dictum of Ponder, "If there is any band of nuclear material connecting the different parts of a nucleus, that nucleus for the purpose of a count cannot be said to be divided."

RESULTS

Table I shows the trend of the nonfilament count before, during, and after excitement. These counts averaged 20.8 in the first normal, 20.5 in the second,

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20.5 in the excited state, 19.7 in the first recovery, and 20.7 in the second recovery. The additional numbers, respectively, necessary to make the 100 counted, were filament cells.

TABLE I

THE NONFILAMENT COUNT BEFORE, DURING, AND AFTER EMOTIONAL EXCITEMENT
30-MINUTE INTERVALS

RABBIT	SEX	FIRST NORMAL	SECOND NORMAL	EXCITED	FIRST RECOVERY	SECOND RECOVERY
103A	F	22	20	20	23	22
103B	F	18	18	17	18	18
104A	M	25	24	25	23	24
104B	M	27	24	23	24	23
105A	M	15	15	18	15	15
106A	M	17	17	15	15	16
110B	F	18	18	18	17	18
115B	F	23	25	27	21	20
116C	F	19	23	17	18	20
120A	M	24	21	25	23	24
Average		20.8	20.5	20.5	19.7	20.6

TABLE II

SUMMARY OF EXPERIMENTS ON NONFILAMENT COUNTS DURING EXCITEMENT

	NO. EXPER.	SEX	1ST NOR.	2ND NOR.	EXCITED	1ST REC.	2ND REC.
Normal rabbits	18	M	18	17	18	19	18
	14	F	19	19	18	18	18
Average			18.4	17.9	18	18.6	18
Splenectomized rabbits	15	M	20	18	19	20	20
	10	F	19	21	20	18	18
Average			19.6	19.2	19.4	19.2	19.2

Table II summarizes the results of our 32 experiments on normal rabbits and 25 experiments on the blood of splenectomized rabbits. In the normal group, the averages were 18.4, 17.9, 18, 18.6, and 18, while in the splenectomized rabbits the averages were 19.6, 19.2, 19.4, 19.2, and 19.2.

These results show that the relation of the number of nonfilament to the filament cells is not altered during excitement. During the recovery from the excited state, this relation is also maintained, indicating that excitement does not produce an immediate stimulation of bone marrow activity, nor is recovery for a period of an hour characterized by an increased number of neutrophiles.

SUMMARY

The products of tissue breakdown which are liberated into the blood stream during excitement (anger, fear, and pain) did not deflect the relation of the nonfilament and filament neutrophiles.

The emotional leucopenia in our rabbits seems to be due to the enmeshing of the neutrophiles in the peripheral capillaries and tissues.

REFERENCES

Danzer, M.: Quart. J. Exper. Physiol. **20**: 141, 1930.
Farley, D. L., St. Clair, H., and Reisinger, J. A.: Am. J. M. Sc. **180**: 336, 1930.
Katz, H. L., and Nice, L. B.: Am. J. Physiol. **107**: 709, 1934.
Nice, L. B., and Katz, H. L.: Am. J. Physiol. **109**: 80, 1934.

A SIMPLE TREATMENT FOR PSORIASIS*

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PSORIASIS is a disease of unknown etiology. It has no particular relation to age, sex, color, race, nor diet. It is not transmitted by the closest family contacts. It is not hereditary. One hundred years ago, as "scaly tetter," it had an extensive pharmacopea. Recent curative agents have been sought in salvarsan, colloidal gold, intramuscular injections of the patient's own blood, sex hormones, typhoid vaccine and chaulmoogra oil. Cause and effect are confused in the study of psoriasis by the so-called "spontaneous cures," associated with bed rest, recovery from other diseases, vacations, change of diet.

A commonly observed fact in the South concerning this disease is that it generally clears up to some extent in the summer sun. This led the author to the hypothesis that it might be cured with viosterol. A trial test was made of the hypothesis. A patient with a case of ten years' standing, continuous duration, was put on a routine treatment of viosterol: two gelatin capsules containing 3 minims each of haliver oil with viosterol, daily. Within sixty days from the beginning of the test, the skin of this patient was entirely clear.

Two selected cases were then tested: one, a nurse at the University Hospital; the other, a colored female patient from the Dermatological Clinic of the University. The two cases were admirable for the test. The first was of only three weeks' duration; the second had run a course of thirty years.

The first case was that of a young, well-nourished white girl who had recently entered the nursing class of the Hospital. About three weeks before our test began, she had "broken out" with a severe psoriasis, showing the typical lesions on arms, legs, and back, with even a few scaly patches on her forehead. She was put on viosterol, two capsules per day. This was continued from February 11 through March and the early part of April, but her condition did not materially improve. Her arms were so spotted that she had to be put to night duty in the wards. For the first month and a half she had no other treatment than the viosterol. Later she tried various standard salves and patented ointments. She did not improve.

In May (20, 1935) the viosterol treatment was increased to a massive dose of ten capsules per day for ten days. At 2,000 units of vitamin D per capsule, this would give a total of 200,000 units for the period. A ten-day rest period was interpolated and the massive dose repeated on June 11. Improvement has been slow, but now she has only a few small inconspicuous patches on the elbows and a few spots on her legs. "Maintainance doses" of two capsules per day are being continued.

The second case was that of a colored female, aged forty-nine years. She has had the disease, according to her own statement, for "thirty years." She

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has had every type of known treatment, including a course of "shots." Her lesions are extensive, of the overlapping fungoid type. Feb. 21, 1935, she was started on the same type of treatment as Case 1, i.e., two capsules of haliver oil with viosterol per day. Benefit was questionable. Sometimes the individual patches were better, sometimes worse. In May she was put on the heavy dosage of viosterol, i.e., 10 capsules per day for ten days, with a rest period of ten days and a repetition of the dosage. During July, her skin cleared up so that "she didn't have a scale." But in August she broke out again. Today, her condition is materially improved over what it was in February. We have put her back on a "massive dose" and will continue on a "maintainance" dose as soon as the condition is under control.

DISCUSSION

Our experience with these three cases of psoriasis leads us to this preliminary report. We realize the shortcomings of such a limited experience in the light of what is now called "experimental research" that demands at least two hundred cases. If the treatment were at all hazardous or difficult, we would not presume to lay it before the profession. But the treatment is so simple that it should be put to a trial test in the interest of every patient suffering from this obnoxious condition. Certainly it is worth a fair trial. We leave our results to be tested on a more elaborate scale by the larger clinics.

We wish to take this opportunity to express our thanks to Dr. J. F. Biehn and Dr. Richard Lucke of the Abbott Laboratories for their generous grants of haliver oil used in conducting these tests. To Dr. Guy Bernard, Professor of Dermatology of the University of Georgia School of Medicine, we are indebted for kindly advice and interest and for the opportunity of working in his clinic.

SOURCES OF ERROR IN THE LABORATORY DIAGNOSIS OF AMEBIASIS*

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DURING the latter part of a survey conducted by the Chicago Board of Health following a recent outbreak of amebiasis in Chicago, it became evident that the use of cultures might increase the difficulties in differentiating the trophozoites of *Endameba coli* and *Endameba histolytica*. A study of this problem revealed interesting, although not conclusive, results. In view of the probable importance of the observations, it is believed that their publication will not be out of place. This report deals only with the diagnosis of amebiasis by culture, by direct smear, and by fixation and staining. No reference is made to the potentially useful complement fixation test, as it was not used in this survey.

The diagnosis of amebiasis is made primarily in the laboratory. Because of this, the identification of *Endameba histolytica* assumes much importance. It is of more importance since it was observed from studies on numerous samples during the recent Chicago epidemic and resultant survey, that *Endameba coli*, a nonpathogenic ameba, might very often be confused with *Endameba histolytica*. Although the fact that *Endameba coli* may sometimes possess some of the characteristics of *Endameba histolytica* was appreciated by Wenyon and O'Connor,[1] Dobell and O'Connor,[2] Hegner, Johnson and Stabler,[3] Stabler,[4] and others, its importance has probably not been fully emphasized.

Since other conditions may simulate amebiasis clinically, such as nonspecific ulcerative colitis, tuberculous enteritis, carcinoma of the colon, functional "colitis," the milder types of bacillary dysentery and other less common conditions, it is feared that the finding of a few nonpathogenic amebae, particularly *Endameba coli*, mistaken for the pathogen, *Endameba histolytica*, may serve to lead the clinician astray as to treatment and prognosis.

It is the problem of differentiating *Endameba coli* and *Endameba histolytica* that is given the greatest consideration in this report. The remaining intestinal amebae should offer the experienced worker little trouble.

DIAGNOSIS BY DIRECT SMEAR

This method, although open to error, is one of the most reliable available, provided certain precautions are observed. It is absolutely necessary that fresh specimens be obtained and if loose should be examined within thirty minutes. Even in formed stools it has been observed that cysts may begin to degenerate in the course of several hours. Refrigeration or incubation does not necessarily guard against degeneration. Specimens containing oil or obtained by oil cathartics are unsatisfactory as are samples containing bismuth or arsenic crystals.

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Slides should be wiped immediately before using, as crystals resembling bismuth may be found on "clean" slides. One to two weeks should have elapsed following a barium enema for roentgenologic purposes, before an examination should be considered satisfactory.

Medicines containing arsenic or bismuth should be discontinued for the week preceding the examination for amebiasis. Where possible, antiamebic drugs should be withheld until sufficient examination has been made either to confirm or to rule out the diagnosis of amebiasis. Such examination should include five to six stool examinations, one of which should be obtained by a rectal tube or proctoscopy. Much valuable time may be lost if bismuth and arsenic preparations are given before a definite diagnosis is made.

The types of amebae found will by cysts, trophozoites and precysts, and what has been called "inactive trophozoites." It is generally agreed that where cysts of *Endameba histolytica* are present, the diagnosis of amebiasis is definite. Occasionally four-nucleated cysts of *Endameba coli* are found which may be confusing, but a study of the cytoplasm, which is very evenly, coarsely granular in *Endameba coli* in iodine stain, and a study of the nuclei, will aid in the diagnosis. Very small cysts of *Endameba histolytica*, ranging in size from 6 to 7μ, sometimes offer difficulty but will cause little trouble to the experienced observer.

It is also agreed that where trophozoites of *Endameba histolytica* are present, which have ingested red cells, the diagnosis is definite. Whereas, samples containing cysts are relatively frequent, the microscopic picture of acute amebiasis accounts for only a small percentage of infections in the temperate zone. This means that, in the temperate zone, one will find very few cases with typical trophozoites containing ingested red cells.

Where trophozoites are present which exhibit a majority of the accepted characteristics of *Endameba histolytica*, the diagnosis of amebiasis is probably also justifiable.

The characteristics which are necessary for a diagnosis of typical trophozoites of *Endameba histolytica*, not containing red cells, are, rapid progressive motility, clear hyaline pseudopodia, either explosive or merely "crawling" along, thin cytoplasmic wall, ectoplasm and endoplasm clearly differentiated, cytoplasm rarely containing bacteria, yeasts, or débris and a thin-walled, evenly granular, indistinct nucleus. The absence of any one of these characteristics should raise considerable doubt as to the diagnosis of amebiasis. The remaining samples, however, will offer much difficulty in diagnosis, since they will contain forms which could be either *Endameba coli* or *Endameba histolytica* or both. Even under the best of conditions, the characteristics differentiating *Endameba histolytica* from *Endameba coli* are not marked. Wenyon,[5] in 1925, in discussing the validity of Councilmania and a comparison of it with *Endameba histolytica* and *Endameba coli*, stated that "it is his opinion that any single species may show all the variations which are supposed to characterize these several species."

That *Endameba coli* may attain an activity comparable to that of *Endameba histolytica*, has been observed by Wenyon and O'Connor[1] and others. In our

experience this was not infrequent and particularly after the administration of a saline purge. Even were *Endameba coli* never actively motile, one could seldom be sure that the ameba in question was not a sluggish *Endameba histolytica*. The remaining characteristics of trophozoites of *Endameba histolytica* are of less differentiating value, although of definite value in forming an impression of the ameba in question, and thereby stimulating further search. However, very often such characteristics are of no help and either fixation and staining are needed or other samples should be obtained in order to arrive at a diagnosis. As many as eight to ten or more samples may be required in some cases to make a diagnosis.

DIAGNOSIS BY CULTURE

Since the introduction of a suitable culture medium by Boeck and Drbohlav,[6] various other media and improvements have been devised by Dobell and Laidlaw[7] Cleveland and Collier,[8] Craig,[9] and others. The addition of rice starch to the original Locke-egg-serum medium, by Dobell and Laidlaw[7] was a decided improvement, for it offered a more luxuriant growth of amebae. Cleveland and Collier[8] devised a liver infusion agar-serum medium, which, with the addition of rice starch, offered an even better growth of amebae. The latter medium was used in the Chicago survey and the following observations are limited to it.

While many studies have been made concerning the comparative value of culture with that of direct smear examinations in diagnosis and varying conclusions reached, several important points must be considered in evaluating such reports. The most important of these are: (1) The number of smears examined from a single sample and the time allowed for the examination of each smear; (2) the experience and ability of the observer; and (3) knowledge of the fact that *Endameba histolytica* and *Endameba coli* cannot always be differentiated in culture. This last point had been noted by Dobell and Laidlaw[10] who state that "the two species are extremely alike in cultures." Doubtless other investigators have realized this fact as well.

When it was realized that *Endameba coli* and *Endameba histolytica* might often be confused in cultures, the following studies were made. An investigation into the procedures used in the preparation of the medium revealed no detectable error. Samples were then obtained which contained only *Endameba coli* cysts and a portion about the size of a walnut was chosen for examination. From ten to fifteen iodine stained smears were made from various parts of the stool in order to be certain that other species were not present. Special attention was paid to the possibility of the presence of small cysts of *Endameba histolytica*. In addition, ten to fifteen smears were fixed and stained and each examined thoroughly. When it was determined that no other amebae were present, a portion of the stool was concentrated and stored in the ice box. The concentrated material usually contained from 10 to 15 cysts per low power field. This was cultured in buffered Locke-serum-starch medium as used by Bishop.[11] Careful and prolonged examination of such cultures after twenty-four and forty-eight hours revealed that of 11 such treated samples, 6 excysted to produce some trophozoites similar to *Endameba histolytica*, 3 produced only *Endameba coli*

trophozoites, and 2 did not excyst. It was impossible in any of the samples to produce encystation after excystation had occurred, but the cultures could only be maintained for four to five subcultures. Studies were also made on liver infusion agar cultures, inoculated with the same concentrated material and similar results were obtained. Subcultures from Locke serum medium to liver infusion medium caused no change in the characteristics of the trophozoites. In addition to these studies observations on many other samples, not however, permanently stained, revealed that such cysts often excysted to produce trophozoites which could not, without fixation and staining, be differentiated with certainty from *Endameba histolytica*.

Many samples, however, contained only sluggish or "inactive" trophozoites without red cells, which could be either *Endameba histolytica* or *Endameba coli* or both. Studies were limited to single infections since little information could be obtained from double infections. Such samples were examined by direct smear, culture, and fixation and staining. Impressions of the direct smear were recorded and examinations of cultures were made at three, twenty-four, and forty-eight hours. Several such cultures were examined at hourly intervals up to twelve hours, and from such studies it was concluded that early examination, that is, before eighteen hours, is a definite source of error. Fifteen samples, containing sluggish or "inactive" trophozoites, were studied as described and the results are recorded in Table I.

TABLE I

COMPARISON BY DIRECT SMEAR, CULTURE, AND STAINED SMEAR IN THE EXAMINATION FOR *Endameba histolytica*

IMPRESSION OF DOUBTFUL TROPHOZOITES BY DIRECT SMEAR		RESULTS OF THE SAME BY CULTURE		RESULTS BY FIXATION AND STAINING	
"*E. coli*"	11	"*E. histolytica*"	8	*E. histolytica*	2
				E. coli	6
		"*E. coli*"	3	*E. histolytica*	1
				E. coli	2
"*E. histolytica*" (Three obtained by saline purge)	4	"*E. histolytica*"	4	*E. histolytica* *E. coli*	0 4
		"*E. coli*"	0		

From a study of the table, it is seen that the majority of the doubtful trophozoites were *Endameba coli*, many of which would ordinarily be designated as *Endameba histolytica* by culture. It is realized that such observations are based on a small series of cases, but from previous experience, it seems likely that such results would be confirmed on a larger series of similarly studied cases. It must be realized that the percentage of error, as suggested by the table, does not hold for routine random samples, since the above samples are selected cases.

From the above observations, it is believed that the use of primary cultures, particularly in surveys, is likely to cause error in the diagnosis of amebiasis, and for that reason, should not be used as a means to a final diagnosis.

DIAGNOSIS BY STAINED SMEARS

It is essential that every laboratory, where the diagnosis of amebiasis is made, should have adequate staining facilities. A very reliable, although a rather laborious staining method is the use of iron hematoxylin after fixation with heated Schaudinn's solution. The staining of amebae requires experience and patience, and this is true also of the diagnosis of such smears.

The characteristics of the stained nuclei of stool amebae are best recorded in the standard textbooks on protozoology and will not be referred to here. Certain precautions are, however, necessary. Where stained smears are necessary, it is essential that the sample, if loose, be stained within thirty minutes. Degenerating nuclei will be very confusing and cannot always be relied upon for diagnosis. It is also necessary that the sample contain an adequate number of amebae, since double infections are common and many amebae will be lost in the process of staining.

The characteristics of the stained nuclei of cultured amebae of *Endameba coli* and *Endameba histolytica* are not as distinguishing as in the nuclei of stool amebae. Martin[12] observed the thickening of the nuclear wall of *Endameba histolytica* that takes place in cultures. Often it is difficult to determine the species by staining. Because of this difficulty the routine diagnosis by staining of cultures is not recommended.

SUMMARY

1. Since the characteristics of trophozoites of *Endameba histolytica* and *Endameba coli* in primary culture are so often very similar and therefore indistinguishable, the use of primary cultures in the diagnosis of amebiasis is a definite source of error and, therefore, should be used with caution as a means to a final diagnosis. (It is probable that future study will find substances lethal to *Endameba coli* while not lethal to *Endameba histolytica*, thereby providing cultures as a very useful aid in diagnosis.)

2. Fixation and staining of trophozoites from cultures is not an accurate means to diagnosis, since the nuclear changes which occur in cultures cause a rather marked similarity between the nuclei of *Endameba histolytica* and *Endameba coli*.

3. The diagnosis of amebiasis by direct smear is certain only when (1) cysts or (2) trophozoites containing ingested red cells or (3) typical trophozites of *Endameba histolytica* are present. In samples containing inactive or sluggish trophozoites, fixation and staining should be used or other samples obtained.

4. For the present, at least, examination of samples should be confined to material from a natural bowel movement. It is quite possible that a saline purge may increase the number of amebiasis cases found on a single examination, but its use is not recommended, since in our experience it may cause confusion. Following a saline purge, *Endameba coli* becomes quite active and tends to assume the characteristics found in the pathogenic form.

The writer wishes to express his appreciation to Dr. William H. Taliaferro for his valuable advice and assistance and to Dr. Herman N. Bundesen for his cooperation in this problem.

REFERENCES

1. Wenyon, C. M., and O'Connor, F. W.: Human Intestinal Protozoa in the Near East, London, 1917, 218 pp.
2. Dobell, C., and Connor, F. W.: The Intestinal Protozoa of Man, London and New York, 1921.
3. Hegner, R., Johnson, C. M., and Stabler, R. M.: Host-Parasitic Relations in Experimental Amebiasis in Monkeys in Panama, Am. J. Hyg. **15**: 1932.
4. Stabler, R. M.: An Extended Study of Variations in a Single Race of Coli-like Amoebae and Its Bearing on the Specificity of Councilmania lafleuri, Am. J. Hyg. **16**: 1, 1932.
5. Wenyon, C. M.: The Genera Councilmania, Karyamoeba and Caudamoeba, Trop. Dis. Bull. **22**: 333, 1925.
6. Boeck, W. C., and Drbohlav, J.: The Cultivation of Endamoeba histolytica, Am. J. Hyg. **5**: 371, 1925.
7. Dobell, C., and Laidlaw, P. P.: On the Cultivation of Entamoeba histolytica and Some Other Entozoic Amoebae, Parasitology **18**: 282, 1926.
8. Cleveland, L. R., and Collier, J.: Various Improvements in the Culture of Entamoeba histolytica, Am. J. Hyg. **12**: 606, 1930.
9. Craig, C. F.: A Simplified Method for the Cultivation of Endamoeba histolytica, Am. J. Trop. Med. **6**: 1926.
10. Dobell, C., and Laidlaw, P. P.: The Action of Ipecacuanha Alkaloids on Endamoeba histolytica and Some Other Entozoic Amoebae in Culture, Parasitology **18**: 206, 1926.
11. Bishop, A.: Experiments on the Action of Emetine in Cultures of Entamoeba coli, Parasitology **21**: 481, 1929.
12. Martin, D. L.: The Lesions of Experimental Amebic Dysentery, Arch. Path. **10**: 349 and 531, 1930.

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AMIDOPYRINE AND THE CIRCULATING LEUCOCYTES*

Effect of Prolonged Administration on the Number and Type of White Blood Cells

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ALTHOUGH Schultz[1] described the disease "agranulocytosis" in 1922, no etiologic factor was established until Kracke[2] in 1932 pointed out the probable implication of various drugs containing a modified benzene ring, prominent among which was amidopyrine. This apparent relationship between amidopyrine and granulopenia was substantiated by Watkins,[3] Rawls,[4] and Madison and Squier.[5] No one asserted that these drugs were the sole cause of granulopenia, yet general opinion was such that Reznikoff[6] felt constrained to warn the profession against leaping to unfounded conclusions on this score. There seems to be little doubt that in certain individuals amidopyrine is capable of causing granulopenia; on the other hand it is universally admitted that the vast majority of patients receiving amidopyrine develop no such blood picture. While the latter statement seems to be self-evident, we were somewhat astonished to find that no actual basis for such a conclusion was revealed in the literature. Fitz-Hugh[7] described a few patients in whom large doses of amidopyrine had

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no demonstrable effect on the circulating leucocytes, but no extensive survey has come to our attention.

With a survey of this kind in mind, 130 patients at the Hamilton County Home and Chronic Disease Hospital were selected for study. Of this number, 103 were followed for an adequate period of time, and these constitute the material on which this paper is based. Patients were selected more or less at random from a heterogeneous hospital population, with some emphasis on those with chronic arthritis, and with the exclusion of those suffering from severe disease of the heart or the blood-forming organs. The majority (79) were in the fifth, sixth, or seventh decades of life, the remainder scattered fairly uniformly in the second, third, fourth, and eighth. Sex and color distribution was as follows: 64 white males, 25 white females, 8 colored males, and 6 colored females. All of these patients received amidopyrine, and in addition a small number (20) of them received isoamylethyl barbituric acid (amytal), as noted below. All blood work was done by two technicians, and all counts in each case were done by the same technician throughout. After three or more preliminary counts during a period of ten to fourteen days, amidopyrine was administered as shown in Table I.

Throughout the course of medication and for one or two weeks thereafter, white and differential counts were made at weekly intervals. Determinations of the suspension stability (sedimentation rate) of the erythrocytes were made in a few (eight) cases at weekly intervals.

In not a single patient did a significant decrease in the number of leucocytes or granulocytes occur. The number of leucocytes and granulocytes fluctuated within rather wide limits in exactly the same manner as in the preliminary counts. In one case (see Table I) an actual leucocytosis, accompanied by nausea and malaise, was provoked by amidopyrine on several occasions; the highest of these counts was 16,000 with 86 per cent neutrophiles; the initial leucocytosis was not followed by neutropenia even when amidopyrine was continued (1 gm. daily) for several weeks; eosinophilia did not occur at any time, and skin tests with amidopyrine were negative.

TABLE I

SCHEDULE OF AMIDOPYRINE ADMINISTRATION

NO. OF CASES	DAILY AMOUNT GM.	NO. OF DAYS	TOTAL AMOUNT GM.
60*	1.0	42	42
	1.0	35	
15	2.0†	7	49
5	1.0	35	35
4	1.0	49	49
8	2.0	21	42
10**	1.0	14	14
1	Various doses (see text)		

*Twenty of this group, after one week's rest, received amytal 0.3 gm. daily for two weeks.

**These patients received amidopyrine for two periods of seven days each, with a ten-day rest between.

†The dosage of 1.0 gm. daily for thirty-five days was followed by 2.0 gm. daily for seven days.

All medication was given in divided dosage, i. e., three times daily.

Twenty patients inadvertently received 3.0 gm. (45 gr.) of amidopyrine daily during the first two days of medication; nausea was provoked in several of them, but no leucopenia occurred.

The sedimentation rate was not affected in any of the eight cases in which this test was performed.

COMMENT

It is evident that no conclusions concerning the incidence of granulopenia in patients taking amidopyrine can be drawn from our experiments, since the number of subjects was comparatively small, and since no cases of granulopenia occurred. Yet, if amidopyrine in itself is capable of "depressing" the bone marrow, it seems that some degree of granulopenia should have occurred in this material. Not only were many of the patients enfeebled by reason of age and illness, but nine of them showed a moderate leucopenia before amidopyrine was given; moreover, many of them were distinctly anemic. According to Roberts and Kracke,[8] they would fall into the class of persons with "poor" bone marrow; yet in the face of intercurrent infection, three of these patients with leucopenia developed a distinct leucocytosis during the administration of amidopyrine.

Since the question of "sensitization" has arisen in connection with granulopenia due to amidopyrine, the drug was administered to a group of ten patients in an intermittent fashion, i.e., a rest period of ten days intervened between two courses of one week each. In addition, many of the patients were known to have received amidopyrine on one or more occasions before this study was begun.

The use of one of the barbiturates was prompted by the belief held in some quarters that this group of drugs is sometimes implicated in the causation of granulopenia. There is, however, very little credible evidence of such a causal relationship (Kracke and Parker[9]).

Since changes in sedimentation rate frequently parallel changes in the leucocyte level, this test was performed in a small group; no definite trend was established.

The blood smears were carefully examined by us in many cases, in order to detect any morphologic alterations such as "toxic granules" in the neutrophiles; no such changes were observed. The differential formula in general was not affected in any consistent fashion.

The entirely negative results of this clinical experiment would seem to substantiate the widely held opinion that granulopenia, when associated with amidopyrine medication, is a matter of individual susceptibility. Except in the presence of some predisposing factor, amidopyrine is probably incapable of causing granulopenia.

SUMMARY

In 103 patients, the majority of whom were in the fifth, sixth, and seventh decades of life, no significant decrease in the number of leucocytes or granulocytes followed the administration of large doses of amidopyrine over extended periods of time.

REFERENCES

1. Schultz, W.: Ueber eigenartige Halserkrankungen, Deutsche med. Wchnschr. **48**: 1495, 1922.
2. Kracke, R.: Experimental Production of Agranulocytosis, Am. J. Clin. Path. **2**: 11, 1932.
3. Watkins, C. H.: Possible Rôle of Barbiturates and Amidopyrine in Causation of Leukopenic States, Proc. Staff Meetings Mayo Clinic **8**: 713, 1933.
4. Rawls, W. B.: Neutropenia Developing During Amidopyrine Medication, Am. J. M. Sc. **187**: 837, 1934.
5. Madison, F. W., and Squier, T. L.: Etiology of Primary Granulocytopenia (Agranulocytic Angina), J. A. M. A. **102**: 755, 1934.
6. Reznikoff, P.: Relation of Amidopyrine and the Barbituric Acid Derivatives to Granulocytopenia, J. A. M. A. **102**: 2183, 1934.
7. Fitz-Hugh, Jr., T.: Drug Idiosyncrasy, With Special Reference to Amidopyrine as a Cause of Agranulocytic Angina, Ann. Int. Med. **8**: 148, 1934.
8. Roberts, S. R., and Kracke, R.: Agranulocytosis, Ann. Int. Med. **5**: 40, 1931.
9. Kracke, R., and Parker, F. P.: Etiology of Granulopenia (Agranulocytosis) With Particular Reference to Drugs Containing the Benzene Ring, Am. J. Clin. Path. **4**: 453, 1934.

A FATAL CASE OF TULAREMIC PNEUMONIA WITH ASSOCIATED ILEITIS*

A Clinical and Pathological Report

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INTRODUCTION.—Although a fair number of cases of fatal tularemic pneumonia have appeared in the literature, reports of intestinal lesions in tularemia are exceedingly rare. Recently a case of tularemia came to our attention which resembled typhoid fever clinically, and at autopsy presented typical tularemic pneumonia and an associated ileitis. Because the latter condition has been such an infrequent finding in tularemia, it was thought worth while to place this case report in the literature.†

Review of Literature.—Pathologic studies in cases of tularemic pneumonia have been reported by several writers. In 1931, Permar and Maclachlan[1] reviewed previously reported cases and added one of their own. They described the histopathology of the pneumonic process and called attention to the lack both of external skin lesions and lymphatic enlargement in their case. The serious prognostic import of lung involvement has been mentioned by Francis[2] and others. Gundry and Warner[3] in 1934 published a review of all autopsied cases of tularemia appearing in the literature up to that time, along with one additional case. They stressed the importance of pulmonary lesions in fatal tularemia. Regarding abdominal complications in tularemia, peritonitis has been observed in four cases, namely those of Schumacher,[4] Foulger, Glazer and Foshay,[5] Gundry and Warner,[3] and Kavanaugh.[6] In the case of Schumacher there was some ulceration of the cecum in addition to the peritonitis. In 1935

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Beck and Merkel[7] reported a case of tularemia with autopsy findings, in which multiple small superficial mucosal ulcers were encountered within the stomach, duodenum, lower third of ileum, and ascending portion of colon. There was neither peritonitis nor pneumonia associated. The disease had been contracted following ingestion of insufficiently cooked rabbit meat.

In the above brief review of the literature no attempt has been made to mention or detail the splendid achievements of Francis in the development of our present knowledge of tularemia.

REPORT OF CASE

History.—W. R., a sturdy male negro, aged thirty-four, whose usual occupation was that of a coal miner, worked one day (November 9) in a downtown market, skinning and cleaning wild rabbits. He felt sick the following day, complaining of severe headache, and left-sided chest pain which he related to his heart. He stayed in bed. Gradually he became worse, and was seen on November 20 by an outside physician who sent him in with a provisional diagnosis of pleurisy.

He was admitted Nov. 21, 1934 to the Pittsburgh City Hospital, complaining of pain in his left chest. He was nearly inarticulate, and a satisfactory history could not be obtained. His temperature was 102.4° F.; pulse, 104; respiration, 24; blood pressure, 130/80. The temperature curve from this time until death was septic in type, running between 98° and 104° F. On physical examination the day of admission, breath sounds were diminished, but there were no signs of pulmonary consolidation. Although searched for carefully, no regional lymph gland enlargement could be detected. The entire skin surface was examined for the presence of an abrasion, fissure, or other possible portal of entry, with negative results. Report of routine urinalysis on admission: Sp. Gr., 1.017; color, amber; transparency, cloudy; reaction, acid; sugar, negative; albumin, 1-plus; micro, many pus cells. Blood Wassermann and Kahn taken the following day were negative. Serum taken November 23 was reported as agglutinating *B. tularensis* in dilutions of 1-80 (National Institute of Health). On November 24, the patient appeared quite toxic, and expectorated bright red blood. Examination of his chest on this day disclosed bronchial breath sounds over the left side, anteriorly and posteriorly, with an occasional fine râle. A dull percussion note was elicited over the left lower chest. On November 26, there were coarse râles over both lung fields, and signs of a "full blown" pneumonia were present over the lower left lobe. A blood culture of this date was negative in seventy-two hours. An x-ray of chest taken November 27 was reported: "Pneumonia of the lower lobe on the left. May also have a pericarditis." A blood culture taken this day was negative in seventy-two hours. Microscopic Widal: "Serum agglutinates living eighteen-hour bouillon cultures of *B. typhosus* in dilution of 1-40; no agglutination of *B. paratyphosus* A or B. (This finding seemed significant at the time, but later when the report of agglutination by *B. tularensis* was received, it was thought to have been a nonspecific type of agglutination.) On November 28, the patient seemed confused; the chest signs were unchanged. On November 30, his general condition was poor; moist râles were heard over the entire chest; heart sounds were of poor quality, and the pulse was feeble and rapid. The patient died on December 2.

Autopsy was performed seventy-two hours later. The body was well preserved, it having been in cold storage in the interim. Serum of blood taken at autopsy was reported as agglutinating *B. tularensis* in dilution of 1-10240, and failing to agglutinate *B. typhosus*, para A and para B (Reported by National Institute of Health).

Autopsy.—Externally there was nothing remarkable. Each lung presented a few easily detached yellowish pink fibrinous adhesions. There were about 100 c.c. of cloudy amber fluid in each pleural cavity. The left lung was heavy and voluminous, weighing 906 gm. and measuring 23 by 12 by 9 cm. Its lower lobe was completely consolidated, presenting a firm, slightly convex, moist, granular, grayish pink cut surface with a fine yellowish white mottling. The pleura overlying the lower lobe was reddened and flecked by a few yellowish

white, friable, plastic fibrinous adhesions. The upper lobe was wet and congested but unconsolidated. The peribronchial glands were enlarged and firm; their cut surfaces presented several small yellowish white areas of necrosis. The right lung weighed 500 gm., and measured 23 by 16 by 6.5 cm.; it felt doughy. The pleural surface was purplish red, and presented a few easily detached yellowish white fibrinous adhesions. When sectioned, much blood-tinged watery fluid welled up from the cut surface. A small, roughly triangular, reddish black zone of infarction was present within the lower lobe. The small branch of the pulmonary artery supplying this area was occluded by the presence of a small, soft, reddish pink crumbly cylindrical thrombus, composed of an agglomeration of embolic chunks of thrombus material. The upper and middle lobes were less edematous than the lower. A subpleural bulla, the size of a golf ball, was present within the upper lobe. The peribronchial glands on this side were enlarged and edematous. The heart was pale and flabby but otherwise not remarkable. The aorta was quite elastic. It presented a few superficial yellowish intimal streaks.

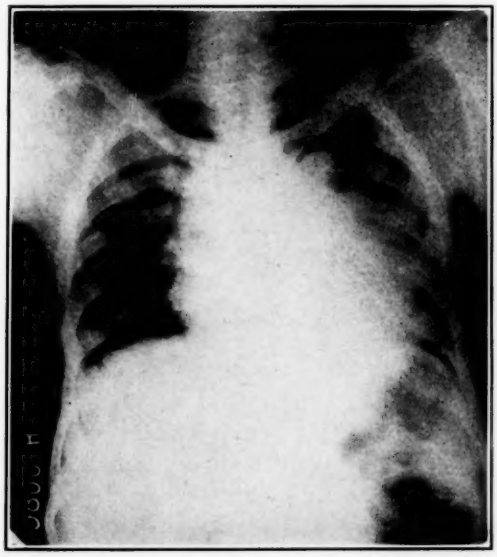

Fig. 1.—Roentgenogram of chest (seventeenth day of disease), showing left-sided pneumonia.

All of the abdominal viscera were normally disposed. The transverse and ascending colon were markedly distended with flatus. The mesenteric lymph glands were swollen and reddened, particularly those in the ileocecal region. There was no peritonitis, but the serous surfaces of the terminal ileum were reddened. The spleen was distinctly enlarged and of unusual firmness. The inferior vena cava was opened at its iliac bifurcation. A small L-shaped antemortem thrombus was found extending into the left iliac vein. This clot presented a reddish pink, granular, finely rippled surface. On opening the gastrointestinal tract, the noteworthy findings were confined to the cecum and terminal ileum. The Peyer's patches in the terminal ileum were prominent and reddened. A few nearest the ileocecal valve presented superficial erosion. These patches appeared raw and hemorrhagic. The mucosa here was generally reddened, as was that of the cecum. The liver was pale and cloudy, but otherwise normal. The gallbladder was negative as was the pancreas. The spleen was firm and enlarged, weighing 330 gm., and measuring 15 by 8 by 5.5 cm. Its capsule was smooth, slate blue, and shiny. The cut surface was deep purplish red, moist, and smooth. No gross foci of necrosis could be detected. The kidneys appeared pale, cloudy, and swollen. The adrenals, bladder, and prostate were not remarkable.

Postmortem bacteriologic studies revealed nothing of interest.

MICROSCOPIC DESCRIPTION

Lungs.—Sections from the left lower lobe presented histologic findings typical of tularemic pneumonia. The alveolar exudate consisted of varying proportions of large mononuclear cells, serum, fibrin, neutrophiles, lymphocytes, erythrocytes, and cellular débris. In some places, the exudate was predominantly cellular, in others, serocellular or serous. The chief exudative cell was a large round or oval mononuclear cell, many representatives of which were

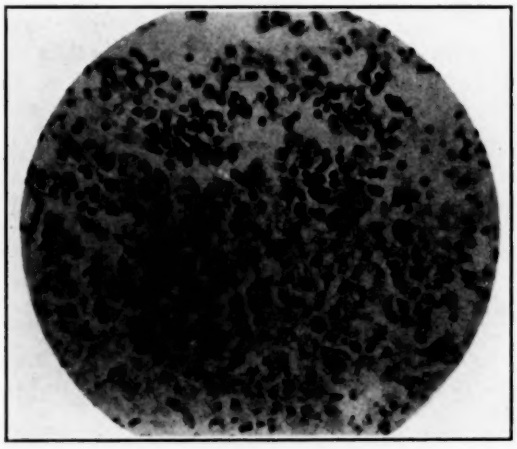

Fig. 2.—Photomicrograph of lung (×560), showing mononuclear alveolar exudate.

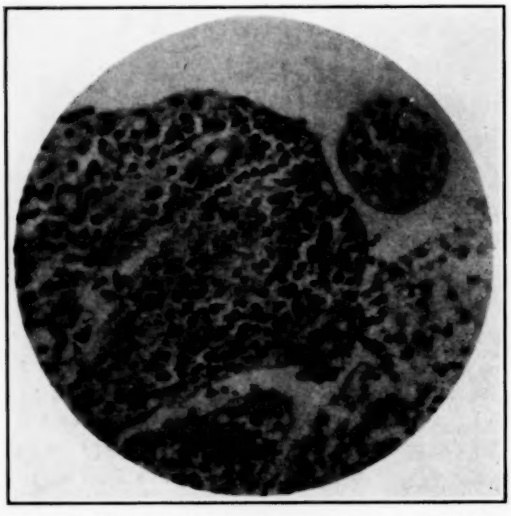

Fig. 3.—Photomicrograph of ileum (×560), showing loss of epithelium, and infiltration of mucosal stroma by large mononuclear cells.

phagocytic. These were present in large numbers; some alveoli were packed with them, others contained only a few. Desquamated alveolar lining cells were scarce. In addition to the alveolar pneumonia, there was a definite interstitial pneumonitis with a mononuclear response. The striking feature of the pneumonia was the presence of large zones of caseous necrosis. Here not only the alveolar exudate, but the alveolar walls as well were necrotic. Several arteries in the vicinity of the caseous foci presented subendothelial edema with thrombosis. The pulmonary tissue in general was moderately anthracotic. Bronchioles within the consolidated area presented a mononuclear mucocellular exudate.

Peribronchial Lymph Glands.—Multiple caseous necrotic foci were seen. These were characteristic of tularemic lymphadenitis. There was moderate anthracosis.

Ileum.—There was superficial erosion, with loss of glandular and lining epithelium. The superficial mucosal stroma was slightly edematous and was studded by large round or oval mononuclear cells. Deeper, there was considerable hypertrophy of the lymphoid tissue with marked reticular cell hyperplasia. The inflammatory reaction was confined to the mucosa.

Mesenteric Lymph Glands.—Sections showed diffuse edema and marked reticular cell hyperplasia within the sinuses.

Spleen.—The pulp was congested. In one section, a microscopic tularemic nodule was noted. Sections of other organs were not of special interest.

COMMENT

The absence of external cutaneous lesions and lymphadenopathy in this patient is of interest. The case from this standpoint is similar to that of Permar and Maclachlan. The portal of entry in both cases may have been the respiratory tract. In our case introduction of infected material into the gastrointestinal tract may have occurred after establishment of the pneumonic process, by the patient's swallowing coughed-up pulmonary secretions. The lesions in the ileum were apparently grossly similar to those in the case of Beck and Merkel, but less extensive. Although the consolidated portion of lung presented histologic features characteristic of tularemic pneumonia, microscopic sections of the ileum were disappointing. There was evidence of a definite ileitis within these slides, but sufficient histologic criteria to establish the ileitis as tularemic were lacking. Despite this absence of conclusive evidence, we believe the ileitis in our case to have been tularemic in nature.

SUMMARY

1. A proved case of fatal tularemic pneumonia is reported with autopsy findings.

2. An associated ileitis is described, which in positive features resembles that reported by Beck and Merkel.

3. No cutaneous site of entry was detected and the possibility of primary respiratory tract invasion is suggested.

REFERENCES

1. Permar, H. H., and Maclachlan, W. W. G.: Tularemic Pneumonia, Ann. Int. Med. **5**: 687, 1931.
2. Francis, E.: Symptoms, Diagnosis and Pathology of Tularemia, J. A. M. A. **91**: 1155, 1928.
3. Gundry, L. P., and Warner, C. G.: Fatal Tularemia; Review of Autopsied Cases With Report of a Fatal Case, Ann. Int. Med. **7**: 837, 1934.
4. Schumacher, H. W.: Quoted by Francis in.[2]
5. Foulger, M., Glazer, A. M., and Foshay, L.: Tularemia; Report of Case, With Post-mortem Observations and Note on Staining of Bacterium Tularense in Tissue Section, J. A. M. A. **98**: 951, 1932.
6. Kavanaugh, C. N.: Tularemia; A Consideration of One Hundred and Twenty-Three Cases, With Observations at Autopsy in One, Arch. Int. Med. **55**: 61, 1935.
7. Beck, H. G., and Merkel, W. C.: Tularemia: Fatal Case of the Typhoid Form Caused by Ingestion of Rabbit: Autopsy Report, South. M. J. **28**: 422, 1935.

THE CAUSE AND SIGNIFICANCE OF THE ELECTRONEGATIVITY OF ACTIVE LIVING TISSUE*

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INJURY to any part of the organism initiates chemical changes which render the injured portion electronegative to the uninjured. Activity likewise initiates chemical changes which cause the active portion to become electronegative to the inactive. The active and injured area corresponds to the negative pole and the inactive and sound part to the positive pole of a battery, and if these two parts or poles be connected an electric current will flow just as is the case when the poles of a battery are connected. Chemical change is the cause of the electric current from a battery, and it is undoubtedly the cause of the action and demarcation currents in living tissue.

ELECTRONEGATIVITY IN INJURED AND CONTRACTING MUSCLE

The following experiments were carried out in an attempt to determine what chemical changes are brought about, by injuring a muscle, that cause the injured area to become electronegative. A gastrocnemius muscle of the leg of a medium-sized frog was carefully removed, skinned, and cut transversely near one end. A pair of nonpolarizable electrodes were prepared and connected with a galvanometer. When the cut or injured end of the muscle was placed against one of the electrodes and the sound surface against the other (see Fig. 1), the galvanometer registered a flow of current of two to four microamperes from the sound surface to the injured end of the muscle. The application of a small amount of a one molar solution of calcium chloride to the cut or injured end of the muscle immediately abolished the electronegativity and the demarcation current, while the subsequent application of a small amount of a 0.05 molar solution of phosphoric acid or disodium phosphate promptly restored the electronegativity and the demarcation current. This observation was confirmed with the use of a number of muscles. It was also found that the application of a one molar solution of barium chloride was as effective as the calcium chloride in abolishing the electronegativity of the injured end of the muscle and the demarcation current, while the application of a one molar solution of magnesium chloride was less effective. The use of weaker solutions of these salts was found to be effective, but the action was slower. The muscles of turtles were also used, and the results were essentially the same as those obtained with the use of frogs' muscles.

Pieces of ammonium molybdate paper were placed on the cut ends of frogs' muscles and moistened with the muscle juices. Upon removal and drying

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of these pieces of paper at 65° C., they turned distinctly yellow, thus showing the presence of phosphate on the injured ends of the muscles. It is assumed that the application of calcium chloride as well as barium chloride abolished the electronegativity and the demarcation current by combining with the electronegative phosphate ions at the injured end of the muscle to precipitate insoluble and non-ionized calcium and barium phosphate. Magnesium chloride was less effective because the magnesium phosphate formed was more soluble and more highly ionized than either the calcium or barium phosphate. Since the removal of the electronegative phosphate ions abolished the electronegativity of injured muscle, and the restoring of these ions restored it, the conclusion is drawn that the electronegativity of injured muscle is due to the electronegative phosphate ions.

The active or contracting portion of a muscle, like the injured area, is electronegative to the noncontracting or resting portion. The contracted area

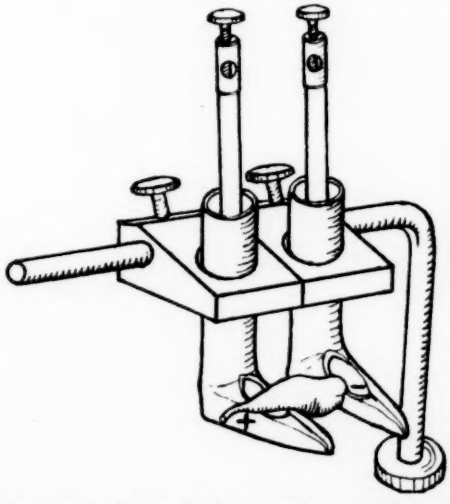

Fig. 1.—Shows injured electronegative end of a muscle against one nonpolarizable electrode and the uninjured electropositive surface against the other.

of the beating heart is electronegative to the resting portion. The phosphorus compounds, creatinphosphate and adenylpyrophosphate, occur in muscle, and it is known that these two compounds are broken down during muscular contraction with the formation of phosphoric acid and liberation of energy for the muscular contraction.[1] The electronegativity of the wave of muscular contraction that sweeps over a muscle upon stimulation like that of the injured area is attributed to the electronegative phosphate ions, arising from the hydrolysis of the creatinphosphate and adenylpyrophosphate during contraction.

The electronegativity produced by mild stimulation is transitory, passes over the muscle and disappears, while that set up by an injury is local and persistent. A very strong stimulus applied to a muscle, sufficiently strong to be injurious, produces, in addition to a transitory wave of electronegativity, a state of persistent and local electronegativity at the site of stimulation. Hence, contracted muscle may be looked upon as slightly injured muscle, and the effect of injury and stimulation is the same; namely, to liberate electro-

negative phosphate ions, and these ions in turn are responsible for the electronegativity of both injured muscle and the wave of muscular contraction.

ELECTRONEGATIVITY IN THE PRODUCTION OF ARTERIOSCLEROSIS

The carotid arteries of an anesthetized dog were exposed, and with the use of a galvanometer and platinum wire electrodes, the electropotential of these vessels was determined. It was found that when two uninjured parts of the carotid artery were connected with the micro-ammeter practically no current flowed, but when an area, either of the exterior or interior of the artery was injured, this area became electronegative to the uninjured, and this electronegativity, like that of the injured muscle, is attributed to the electronegative phosphate ions. The deposition of calcium phosphate in the walls of the arteries to produce calcification or hardening of these vessels is attributed to the combination of the electronegative phosphate ions at the site of the injury with the calcium in the circulating blood. This mechanism of calcification offers an

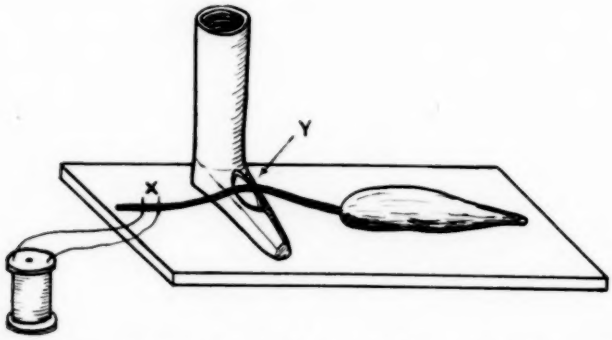

Fig. 2.—Calcium chloride applied to the nerve at "y" blocked the electronegative wave set up by the stimulation of the nerve at "x" and prevented the muscle from contracting.

explanation for the fact that the calcium deposited in the walls of the arteries is in the form of phosphate, that this deposition begins in the intima and is initiated by an injury resulting from infection, strenuous work, etc.

ELECTRONEGATIVITY IN BRAIN AND NERVE ACTIVITY

The brain of an anesthetized dog was exposed and a small area injured. With the use of a galvanometer, it was found that this injured area was electronegative to the uninjured surface or cortex of the brain, and the demarcation current was around 0.5 microamperes. The application of a few drops of 0.1 molar solution of calcium chloride to the damaged brain tissue caused the immediate disappearance of the electronegativity and demarcation current and the subsequent application of a small amount of 0.05 molar solution of phosphoric acid promptly restored the electronegativity and demarcation current. This observation was confirmed with the use of a number of dogs. The use of ammonium molybdate paper showed the presence of phosphate on the injured area of the brain. Hence, the electronegativity of the damaged brain tissue, like injured muscular tissue, is attributed to the electronegative phosphate ions.

The gastrocnemius muscle with its nerve attached was removed from the leg of a frog and prepared for stimulation (see Fig. 2). When the nerve was stimulated, waves of electronegativity were set up in the nerve, and these passed to the muscle causing waves of electronegativity and contraction in the muscle. A small amount of a one molar solution of calcium chloride was applied to the nerve between the point of stimulation and the muscle, and it was found that the muscle would still contract upon stimulation of the nerve. The sheath of the nerve was then broken between the point of stimulation and the muscle with the use of a fine needle, and the stimulation of the nerve still caused the muscle to contract. But upon the application of a 0.5 molar solution of calcium chloride to the nerve where the sheath was broken, stimulation of the nerve failed to elicit a contraction of the muscle. This observation was confirmed with the use of several nerve-muscle preparations.

Fig. 3.—Shows platinum wire electrodes on the brain and on the sciatic nerve preparatory for stimulation and connection with galvanometer.

It is known that phosphorus compounds are an essential constituent of nerves and nervous tissue, and the blocking of the wave of electronegativity by the application of calcium chloride is attributed, as in the case of the injured muscle and brain, to the reaction of the calcium with the negative phosphate ions to precipitate insoluble and non-ionized calcium phosphate. The nerve impulse may accordingly be regarded as a wave of propagated electronegative phosphate ions that passes over a nerve similar to the wave of propagated electronegative phosphate ions that passes over a muscle with the wave of contraction.

The brain and sciatic nerve of a frog were exposed and platinum electrodes placed on the brain and nerve (see Fig. 3). The electrodes on the brain

were connected with an inductorium preparatory for stimulation, and those on the sciatic nerve were connected with a galvanometer. Stimulation of the motor area of the brain set up waves of electronegativity or nerve impulses which passed down the spinal cord out over the motor nerves to the muscles and caused them to contract with a resulting kick of the foot. The electronegative waves that passed over the nerve were detected with a galvanometer. The electrodes were removed from the nerve and placed on the muscle, and the electronegative waves that passed over the muscle were also detected when the motor area was stimulated. The application of a one molar solution of calcium chloride to the sciatic nerve where its sheath had been broken was found to block these waves of electronegativity, and the stimulation of the motor area of the brain no longer caused a contraction of the muscles of the leg. This observation was confirmed with the use of several frogs.

The method of procedure of the preceding experiment was then reversed. The galvanometer was connected to the electrodes on the brain and the inductorium to the electrodes on the sciatic nerve. The object of the experiment was to determine if the stimulation of the sciatic nerve would produce any change in the electropotential of the cortex of the brain and if electronegative phosphate ions were involved in the production of this electrical change.

So far as stimulation of sensory nerves going to the brain producing a change in the electropotential of the brain is concerned, many experiments are reported in the literature, showing that this can be done. Bartley and Bishop[2] found that the stimulation of the nerves going from the eyes to the brain produced a change in the electropotential of the center for vision, the area of the cortex of the brain to which the nerves from the eyes run. Lorente de No[3] showed that the stimulation of the nerves to the muscles of the eye changed the electropotential of the medulla, the portion of the brain where these nerves originate. Caton[4] found that there was normally a continuous fluctuation in the electropotential of the cortex or surface of the brain, resulting presumably from cortical activity or the inflow of nerve impulses over sensory nerves from the outlying parts of the body. With the use of the oscillograph, much work is being done at this time on the electrical changes that occur in the brain.[5] What these electrical changes signify or what their relation to cortical activity is or their cause so far as we are aware is unknown. However, it would seem reasonable to assume that, during brain activity, the area or areas of the cortex involved would become electronegative like all other active tissue, and our observations suggest that the cause of this electronegativity is the electronegative phosphate ions. Phosphorus is certainly very essential for muscular activity, and it may be equally as essential for brain activity. So there may be after all some scientific basis for the view formerly held that phosphorus rich foods promote mental activity.

ELECTRONEGATIVITY IN AGEING

Calcium salts are normally deposited, particularly during the period of growth, in the bony tissues to give rise to the hardness and rigidity of the mature bone. With advance in age, calcium salts are also deposited in certain of the soft tissues of the body. The crystalline lens of the eye gradually loses

its elasticity as one grows older, due to the deposition of calcium salts, resulting in a gradual receding of the near point of distinct vision and loss in the power of accommodation. At twenty years of age a normal person possesses 10 diopters of accommodation, but at the age of sixty all this has been lost, and this loss has occurred at the uniform rate of ¼ diopter per year. So uniformly and constantly does this loss in the power of accommodation occur, as calcium salts accumulate in the lens, that the age of a person may be ascertained with a high degree of accuracy by determining the near point of distinct vision. With the accumulation of very large amounts of calcium salts in the crystalline lens senile cataract results.[6] It is known that ultraviolet is absorbed by the crystalline lens, and its slow calcification with advance in age is attributed to the injurious effect of the short wave lengths of the spectrum, with resulting liberation of electronegative phosphate ions which combine with calcium to produce calcification.[7]

The bones of elderly people become brittle and the joints stiff because of the deposition of calcium salts. Likewise the arteries lose their elasticity and become more or less rigid tubes in the aged, and longevity has come to be considered by many as a vascular question, expressed in the idiom that "a man is only as old as his arteries." No attempt will be made to review the literature on the various phases of calcification, because such reviews have been made recently by Ham,[8] Thomson and Collip,[9] Kay,[10] and Barr.[11] Several theories have been advanced in attempts to explain the mechanism of calcification of the soft tissues of the body. It is known that alkalinity favors the precipitation of calcium salts in vitro, and Hofmeister[12] suggested that the calcification of the arteries might be due to the decreased carbon dioxide tension of arterial blood with resulting increase in alkalinity. He also suggested that the tendency of the alveoli of the lungs, uriniferous tubules, and gastric glands to calcify was due to the alkalinity of these structures brought about by the elimination of acids at these sites. Kleinmann[13] found that dead tissues and necrotic areas were alkaline in reaction, and he attributed their tendency to calcify to this fact. It is recognized that fatty degeneration frequently precedes the calcification of the soft tissues, and according to Klotz,[14] the formation of calcium soaps, which are subsequently transformed into calcium phosphate and calcium carbonate, constitutes the mechanism of calcification. Wells[15] has presented evidence which would seem to indicate that the theory of Klotz regarding calcification is untenable. According to Robison,[16] normal ossification is brought about by the enzyme phosphatase. It is recognized that the presence of vitamin D is necessary for normal ossification, and evidence has been presented to show that large doses of viosterol, as well as of parathormone, may bring about calcification of the soft tissues.[17]

Ageing is attributed to slow continuous calcification of the soft tissues, such as the arteries and the crystalline lens with gradual impairment of function. The stress and strain of the activities of life result in minor injuries to the tissues and organs. These injured areas like the injured muscle and brain are electronegative because of the presence of the electronegative phosphate ions. Calcification results from the combination of these electronegative phos-

phate ions with the electropositive calcium ions in the liquids of the body to precipitate insoluble and non-ionized calcium phosphate at the site of the injury.

Modern medical science has prolonged the average span of life by improving conditions so that more people live to be older, but the upper age limit still remains unchanged. The fact that the doctor lives no longer than his patient is evidence that medical science has done nothing toward solving the problem of senescence. If ageing is essentially a process of calcification of the soft tissues, then senility is an incurable disease, and the prolonging of the upper age limit is an impossibility. For life implies activity with resulting injury, liberation of electronegative phosphate ions and calcification. Hence, with the increase in the strain and activity of our modern civilization, the upper age limit is more likely to be lowered than raised.

SUMMARY AND CONCLUSIONS

1. Since the removal of the electronegative phosphate ions abolishes the electronegativity of injured muscle, and the restoring of these ions restores it, the conclusion is drawn that the electronegativity of the injured muscle is due to the electronegative phosphate ions. The electronegativity of the wave of muscular contraction that sweeps over a muscle upon stimulation is also attributed to electronegative phosphate ions arising from the hydrolysis in the contracting muscle of creatinphosphate and adenylpyrophosphate.

2. The electronegativity of an injured area of the brain like the injured muscle can be abolished by the precipitation of the electronegative phosphate ions with calcium chloride and this electronegativity can be restored by restoring these ions. Hence, the electronegativity of the injured brain is attributed to the electronegative phosphate ions. The waves of electronegativity that pass over a nerve upon stimulation can be blocked by applying calcium chloride to the nerve where its sheath has been broken. These waves of electronegativity are considered to be due to propagated electronegative phosphate ions and the blocking by calcium is attributed to the precipitation of the phosphate as insoluble and non-ionized calcium phosphate. These observations suggest that the change produced in the electropotential of the cortex of the brain by the stimulation of sensory nerves may be due to electronegative phosphate ions, just as the change produced in the electropotential of the muscle by the stimulation of motor nerves is probably due to these electronegative phosphate ions

3. Injury to the intima of arteries was found to render the injured area electronegative to the uninjured portion of the artery. The electronegativity of the injured intima, like the injured skeletal muscle and brain, is attributed to electronegative phosphate ions. It is assumed that the calcification of the arteries is due to the combination of the electronegative phosphate ions at the site of the injured intima with the electropositive calcium ions of the circulating blood. This mechanism of calcification accounts for the fact that the calcium deposit in the walls of the arteries consists almost entirely of calcium phosphate, that the deposition begins in the intima and that it is initiated by an injury. The same mechanism is offered as an explanation for the gradual deposition of calcium in the crystalline lens of the eye. The injury of the lens is attributed to the absorbed short wave lengths of the spectrum.

4. Ageing is considered to be due to a gradual process of calcification of the soft tissues particularly the arteries with resulting impairment of function. Electronegative phosphate ions are liberated as a result of the stress and strain of the activities of life and subsequent calcification is attributed to the reaction of the electronegative ions with the electropositive calcium ions of the liquids of the body to precipitate insoluble calcium phosphate at the site of the injury.

REFERENCES

1. Eggleton, P., and Eggleton, G. P.: A Possible New Factor in the Chemical Mechanism of Muscular Contraction, Chem. & Ind. **46**: 485, 1927.
   Eggleton, P., and Eggleton, G. P.: The Inorganic Phosphate in a Labile Form of Organic Phosphate in the Gastrocnemius of a Frog, Biochem. J. **21**: 190, 1927.
   Fiske, C. H., and Subbarow, Y.: The Nature of the "Inorganic Phosphate" in Voluntary Muscle, Science **68**: 401, 1927.
2. Bartley, S. H., and Bishop, G. H.: The Cortical Response to Stimulation of the Optic Nerve in the Rabbit, Am. J. Physiol. **103**: 159, 1933.
3. Lorente de No', R.: Antidromic Impulse and Response of Mononeurones, Am. J. Physiol. **112**: 595, 1935.
4. Caton, R.: The Electrical Currents of the Brain, Brit. M. J. **2**: 278, 1875.
5. Garceau, E. L., and Davis, H.: An Amplifier, Recording System, and Stimulating Devices for the Study of Cerebral Action Currents, Am. J. Physiol. **107**: 305, 1934.
   Jasper, H. H., and Carmichael, L.: Electrical Potentials From the Intact Human Brain, Science **89**: 51, 1935.
   Davis, H., Derbyshire, A. J., Lurie, M. H., and Saul, L. J.: The Electrical Response of the Cochlea, Am. J. Physiol. **107**: 311, 1934.
   Bishop, G. H.: Fiber Groups in the Optic Nerve, Am. J. Physiol. **106**: 460, 1935.
   Blair, E. A., and Erlanger, J.: A Comparison of the Characteristics of Axons Through Their Individual Electrical Responses, Am. J. Physiol. **106**: 524, 1933.
   Adrian, E. D., and Buyterdijk, F. J. J.: Potential Changes in the Isolated Brain Stem of the Goldfish, J. Physiol. **71**: 121, 1931.
6. Burge, W. E.: Analysis of the Normal and the Cataractous Lens, Arch. Ophth. **38**: 435, 1909.
7. Burge, W. E.: The Mode of Action of Ultra Violet Radiation in Injuring Living Cells With Special Reference to Those Constituting the Eye, Am. J. Physiol. **39**: 335, 1916.
8. Ham, A. W.: Mechanism of Calcification in the Heart and Aorta in Hypervitaminosis D, Arch. Path. **14**: 613, 1932.
9. Thomson, D. L., and Collip, J. B.: The Parathyroid Glands, Physiol. Rev. **12**: 309, 1932.
10. Kay, H. D.: Phosphatase in Growth and Disease of Bone, Physiol. Rev. **12**: 384, 1932.
11. Barr, D. P.: Pathological Calcification, Physiol. Rev. **12**: 593, 1932.
12. Hofmeister, F.: Über Ablagerung und Resorption von Kalksalzen in den Geweben, Ergebn. d. Physiol. **10**: 429, 1910.
13. Kleinmann, H.: Experimentelle Verkalkung durch Zufuhrung von Kalksalzen, Biochem. Ztschr. **196**: 161, 1928.
14. Klotz, O.: The Process of Pathological Calcification, J. Exper. Med. **7**: 633, 1905.
15. Wells, H. G.: Metastatic Calcification, Arch. Int. Med. **7**: 721, 1911.
16. Robison, R.: The Possible Significance of Hexosephosphoric Esters in Ossification, Biochem. J. **17**: 286, 1923.
17. Hueper, W.: Metastatic Calcifications in the Organs of the Dog. After Injections of Parathyroid Extract, Arch. Path. **3**: 14, 1927.

SOME OBSERVATIONS ON GASTRIC ANACIDITY IN RELATION TO GASTRODUODENAL AND COLONIC FLORAS AND AN ASSOCIATED ANEMIA*

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IT IS well known that ordinarily the recurring acid secretion of the stomach presents, directly or indirectly, an effective barrier to the further progress of living ingested bacteria. The content of the normal resting stomach, three to four hours after a meal, is usually sterile although great numbers of bacteria carried in the secretions of the nasal and oral cavities are constantly swallowed together with those in food. The flora of the duodenum[1-4] under normal conditions has also generally been reported as scanty and largely confined to such types as enterococcus, staphylococci, sarcinae with rarely coliform organisms. According to Van der Reis,[5] Goldman,[6] and others, this scarcity of bacteria continues into the jejunum to a distance up to 25 to 80 inches. This flora seems also to be an obligate and persistent type,[6] and not appreciably modified by many bacteria richly varied in type which are continually entering the stomach with swallowed material. On the other hand, with a partial or complete failure of gastric acidity, a rich flora of a mixed buccal and cecal type tends to establish itself.[7] The experimental results of Arnold and Brody[8] indicated that a persistent alkalinity of the duodenum due to the absence in gastric anacidity of the recurrent waves of acidity with normal gastric functioning, and the consequent functional failure of the self-disinfective mechanism of the upper part of the normal small intestine[17] are the factors of primary importance in permitting a bacterial upgrowth from the cecum and thus establishing the rich and varied duodenal flora frequently associated with gastric anacidity.

The observations reported here were carried out primarily to determine whether with the stomach eliminated as a defense mechanism because of failure of acid secretion, the duodenal flora is quantitatively or qualitatively different from the gastric in those cases uncomplicated by colonic flora upgrowth. Do the bacteria swallowed with the oral secretions find their way freely and in a viable condition into the small intestine? If not, to what degree are oral bacteria eliminated in the duodenum, and what types are particularly affected? Although there have been numerous reports on the character of the duodenal flora in the absence of gastric acidity, and it has been shown by Kopeloff[9] and others that the gastric flora under conditions of subnormal acidity is essentially the same as that of the saliva, the relation in types and numbers of viable bacteria of the duodenal to the gastric flora and of the colonic flora to the duodenal, under such circumstances, has apparently not been studied in any great detail. Enquiry

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was also made into possible correlations of degrees of anemia and types of duodenal flora and into the constancy in type of duodenal floras.

The patients investigated, all adults, were in attendance at the Cornell Gastro-Enterology Clinic. All 26 cases had given negative tests for free acid in the gastric content after an Ewald test but had not been given the histamine test for complete achlorhydria. Hence, they are all grouped as gastric anacidity cases.

METHODS

Specimens from each patient for laboratory examination included gastric content, duodenal content, stool and often oral secretions. The patients were instructed to take 1 gr. tablet of phenolphthalein the night before, to brush teeth thoroughly on arising and to present themselves at the office with a stool specimen at about 9:00 A.M. after a 14- to 15-hour fast. In a number of the cases, well-mixed saliva specimens were then collected, and in all, the throat and mouth were thoroughly treated with an antiseptic solution by gargling and rinsing followed by sterile water. A freshly sterilized duodenal tube was then swallowed and permitted to pass to the greater curvature of the stomach. In obtaining specimens from 15 of the cases, the bucket of the tube was covered with a collodion membrane, using the technic of Freeman and Miller[10] with the purpose of preventing contamination. After the bucket had reached the stomach position, the membrane was ruptured by air pressure, the content aspirated, the stomach thoroughly washed with sterile water, and the tube withdrawn. Another tube with covered bucket was then swallowed and the passage into the duodenum assisted by swallowing four ounces of sterile water. When fluoroscopy had shown the bucket in the third part of the duodenum, the membrane was ruptured and the duodenal content aspirated. This technic had the theoretical advantage of minimizing contamination of the duodenal specimen with the gastric flora, but on the other hand was time-consuming and trying to the patient. Experience, in fact, indicated that if the stomach after obtaining the specimen, was thoroughly washed with sterile water, a weak antiseptic solution, and then sterile water until the returning solution was clear, the same tube could then be passed into the duodenum with as little likelihood of contamination by stomach content as with the two-tube method. This single-tube method was used in Cases 16 to 25, and the findings were essentially like those obtained with the two-tube method (Cases 1 to 14). The factor of swallowed saliva could not be controlled entirely under either technic, but the findings indicated it did not seriously affect the purpose of the experiments.

Reaction and cultural examinations were started shortly after obtaining the specimens. These were kept chilled in the interim. The methods employed have been described in detail elsewhere.[11] For reaction determination, 0.1 c.c. of gastric or duodenal material was added to 1 c.c. of diluted bromthymol blue and also phenol red and comparison made with known standards. For aerobic organisms bromeresol purple lactose agar and blood agar plates were surface seeded, for the lactic acid group poured whey agar plates were made and for *Cl. welchii* and other spore-bearing anaerobe determinations cooked minced meat tubes were seeded with graded dilutions with subsequent plating of the growth.

The putrefactive propensity of the whole flora of the stool was estimated according to the method of Leonard and Feirer[12] slightly modified. This method, however, serves as a quantitative gauge solely of the activity of proteolytic spore-bearing anaerobes.

REACTION OF SPECIMENS

In a group of 21 of these gastric anacidity cases, the average reaction of the gastric content was pH 7.2 with ranges from pH 6.8 to 8.0 and for the duodenal content, pH 7.3 with ranges from 6.8 to 7.9. This finding for the duodenum is very close to that reported by Knott[13] for 15 achlorhydria cases, i.e., an average of pH 7.4 with a minimum of 7.0 and a maximum of 7.8. The nature of the flora, according to our findings, seemed to exert little, if any, influence on the reaction, as in 10 cases with numerous coliform types in the gastric and duodenal content the average findings were 7.15 and 7.3, respectively, whereas in 10 cases with weakly acid-producing streptococci and no *B. coli* the averages were 7.3 for the gastric and 7.4 for the duodenal contents.

TYPES OF FLORA IN GASTRIC AND DUODENAL CONTENT

It is well known that in the absence of gastric acidity the flora of the gastric content is much the same as that of the mouth secretions (streptococci of various types, *L. acidophilus*, yeasts, gram-negative cocci). In addition, there may be representatives of the colonic flora (*B. coli*, *Cl. welchii*, and other spore-bearing anaerobes, enterococci). In five cases, second examinations of the gastric content were made at an interval ranging from seven to seventy-nine days from the first. The findings indicated a marked constancy and persistence of bacterial types in the stomach content, in spite of the fact that two patients had had acid treatment in the interim. Quantitatively the gastric flora is generally rich; in this series it was so in 22 cases with relatively few bacteria in only 4 cases. In 15 cases or 57.7 per cent, there were numerous coliform types in the gastric flora, and hence evidence of the upgrowth of the colonic flora: *B. coli communis* in 3, *B. coli communior* in 4, *B. lactici acidi* in 2, *B. aerogenes* in 1, mixtures of communis and lactici acidi in 1, and communior and aerogenes in 1. In 12 (80 per cent) instances, the same *B. coli* types were present in the gastric and duodenal content as in the stool, and in 3 (20 per cent) they were different. In 14 of the 26 cases, staphylococci were found in the gastric or duodenal content; in 12, *Staphylococcus aureus*, and in 2, *Staphylococcus albus*. They were apparently generally of oral origin. *L. acidophilus* was present in the gastric content in 22 of the cases and without doubt mainly of oral origin. *Cl. welchii* was definitely identified as present in gastric and duodenal content in 4 cases and was probably present in three others. As these positive findings were in each instance associated with high *Cl. welchii* counts for the stool specimens and generally with *B. coli* in the gastric content their presence was probably due to upgrowth of the cecal flora.

In 14 or 54.5 per cent of the cases the standardized suspension of stool[11] was positive for *Cl. welchii* up to 10^9 dilution, in 5 or 19.2 per cent for the 10^5 to 10^7 dilutions and in 7 or 26.3 per cent to only 10^1 to 10^3 dilutions; the last is no higher than the usual findings for individuals with a normal gastric acidity.

Moensch, Kahn, and Torrey[14] and others, in a study of the intestinal flora in pernicious anemia, have noted unusually high counts for *Cl. welchii* in the stool specimens. Nye[15] later reported that equally high counts for *Cl. welchii*, as revealed by spore counts and cultures of stools, were associated with gastric achylia in patients not suffering from pernicious anemia. He ascribed these high counts to the tendency of *Cl. welchii* to form spores in the supposedly more alkaline intestinal environment associated with gastric achylia, and hence to assume a more readily recognized form and not to any actual increased growth. The spore estimations for stool specimens of his 5 cases of achylia gastrica, as compared with that for 6 normal individuals as given in his tabulations, indicate an average of approximately 250,000 times as many in the former as the latter. In 14 of our gastric anacidity cases (54.5 per cent), uncomplicated by pernicious anemia, the estimations for *Cl. welchii* (spore stage) were approximately one million times greater than that we have found usual for stool specimens from individuals with a normal gastric acidity, and in 5 cases (19.2 per cent), the counts were from 100 to 10,000 times greater. On the other hand, in 7 cases (26.3 per cent) the counts were approximately the same as for normal individuals. In the four cases which showed *Cl. welchii* in the gastric or duodenal content or both, the *Cl. welchii* counts for the stools were high; in three of these cases there was evidence of upgrowth of the colonic flora (*B. coli*).

There was no evidence that absence of gastric acidity was particularly conducive to putrefactive conditions in the large intestine due to spore-bearing anaerobes. In 14 cases, or 56 per cent, the indications of putrefaction were absent or no more than slight; in 6, or 24.4 per cent, only moderate, and in only 5, or 20 per cent, marked. This is about what might be expected in a series not selected for anacidity. Torrey and Schwartz[16] for 30 cases of psoriasis reported that in 77 per cent the indication of putrefaction was slight or negative, in 10 per cent moderate, and in 14 per cent marked. Leonard and Freirer,[12] in a series of 127 adults not selected for gastric anacidity, found no or slight evidence of putrefaction in 50 per cent, in 33 per cent no more than moderate, and in about 20 per cent marked putrefactive tendencies.

No correlation was found between high *Cl. welchii* counts and evidence of putrefactive propensities of the colonic floras, but rather the contrary. In 11 or 79.9 per cent of the cases showing no more than slight evidence of putrefaction, the *Cl. welchii* counts were particularly high.

THE RELATION OF THE GASTRIC TO THE DUODENAL AND COLONIC FLORAS IN THE ABSENCE OF GASTRIC ACIDITY

In Table I, the findings for 11 cases of gastric anacidity are given in which a simultaneous bacterial survey was made for the mouth secretions, the gastric, the duodenal, and the colonic (stool) contents with purpose of determining what differences, quantitative and qualitative, may appear in the duodenal as compared with the gastric flora and to what extent, as regards numbers and types, mouth organisms under such conditions survive the passage through the entire gastrointestinal tract. Particular attention was given to the gram-posi-

TABLE I

CASE	SEX AND AGE	MOUTH FLORA	GASTRIC FLORA	DUODENAL FLORA	COMPARISON OF DUODENAL WITH GASTRIC FLORA	COLONIC FLORA (STOOL)	PUTREFACTIVE PROPENSITY
12. T.	M 29	Streptoc. (gamma) L. acidoph.	Streptoc. (gamma and alpha) Staph. aureus L. acidoph.	Streptoc. (gamma) Staph. aureus	75% decrease (Streptoc.)	B. coli *Streptoc. (gamma)** Enterococcus L. acidoph. Cl. welchii	None
13. S.	M	Streptoc. (alpha prime, beta) Enterococcus Staph. aureus	Streptoc. (alpha, alpha prime, gamma) Enterococcus Staph. aureus	Streptoc. (gamma) Enterococcus	90% decrease (Streptoc. alpha and Staph.)	B. coli (lactici acidi, aerogenes) Enterococcus L. acidoph. Cl. welchii	Moderate
14. C.	M 43	Streptoc. (alpha and gamma) Enterococcus	Streptoc. (alpha, alpha prime, gamma) Enterococcus B. coli (communis) L. acidoph. Cl. welchii	Streptoc. (gamma) Enterococcus B. coli (communis) L. acidoph.	60% decrease (Streptoc. alpha, alpha prime)	*Enterococcus* *Streptoc. (gamma)* B. coli (communior) L. acidoph. Cl. welchii	None
15. B.	F 49	Streptoc. (alpha, gamma)	Streptoc. (alpha, alpha prime, gamma) Staph. aureus Cl. welchii	Streptoc. (alpha prime, gamma) Staph. aureus L. acidoph. Cl. welchii	90% decrease (all types except Cl. welchii)	B. coli Enterococcus L. acidoph. Cl. welchii	None
16. C.	M 54	Streptoc. (alpha, alpha prime)	Streptoc. (alpha, alpha prime) L. acidoph.	Streptoc. (alpha, gamma) Enterococcus	99% decrease (Streptoc.)	B. coli Enterococcus Cl. welchii	Slight

*Buccal organisms surviving in the colon are italicized.

TABLE I—CONT'D

CASE	SEX AND AGE	MOUTH FLORA	GASTRIC FLORA	DUODENAL FLORA	COMPARISON OF DUODENAL WITH GASTRIC FLORA	COLONIC FLORA (STOOL)	PUTREFACTIVE PROPENSITY
17. R.	F 65	Streptoc. (alpha, gamma) Enterococcus B. coli (communior)	Streptoc. (alpha) B. coli (lactici acidi) L. acidoph.	Streptoc. (alpha, alpha prime) Enterococcus B. coli (lactici acidi) L. acidoph.	50% decrease (Streptoc.)	B. coli (lactici acidi) *Enterococcus* L. acidoph. Staph. aureus Cl. welchii	Moderate
18. O.	F 32	Streptoc. (alpha, gamma) Enterococcus	Streptoc. (alpha, gamma) B. coli (communis) Enterococcus	Streptoc. (alpha, gamma) B. coli (communior) Enterococcus	No decrease No change in flora	B. coli (communis, communior, lactici acidi) *Enterococcus* Cl. welchii	None
19. M.	F 37	Streptoc. (gamma) Enterococcus	Streptoc. (alpha, gamma) Enterococcus Staph. albus B. coli (communis)	Streptoc. (gamma) Staph. aureus B. coli (communis, communior)	80% decrease (Streptoc.)	B. coli (communis, communior, lactici acidi) B. aerogenes *Enterococcus* Cl. welchii	None
23. M.	M 69	Streptoc. (gamma) Enterococcus	Streptoc. (alpha) Enterococcus	Streptoc. (alpha, gamma) Enterococcus	No decrease	B. coli *Enterococcus* L. acidoph. Cl. welchii	None
25. K.	F 48	Streptoc. (alpha) Enterococcus	Streptoc. (alpha) Staph. aureus Enterococcus B. mucosus	Enterococcus B. mucosus	99% decrease (Streptoc., Staph.)	B. coli B. mucosus Streptoc. Cl. welchii	None
26. F.	M 55	Streptoc. (gamma) Enterococcus	Enterococcus B. coli (communior) L. acidoph.	Streptoc. (gamma) Enterococcus B. coli (communior)	50% decrease (Streptoc.)	*Streptoc. (gamma)* Enterococcus B. coli (communior) Cl. welchii	Slight

tive organisms (chain-forming streptococcus and staphylococcus), as they were most likely to be of oral origin. In 9 of these 11 cases (81.8 per cent), there was noted a very marked quantitative decrease when the duodenal flora was compared with the gastric. This was due to the partial or complete elimination of streptococcal types (alpha, alpha prime and gamma) of oral origin, pneumococcus when present, and to some extent staphylococci. Including the whole series of 25 cases, in 18 (72 per cent) the duodenal bacterial counts were 50 to 90 per cent lower than the gastric and in 9 (36 per cent) the reduction amounted to 90 per cent or more. In 7 cases (28 per cent), there was no quantitative difference.

In a few cases, the duodenal flora was entirely different from that of the stomach. When *B. coli* was present in the stomach, it was found in equal or greater numbers in the duodenum and almost invariably the same type. The most marked differences in the gastric and duodenal floras were in streptococcal types. Those of the gastric content were, as might be expected, practically identical with those of the buccal secretions, but in most instances the alpha and alpha prime chain-forming types, prominent in the gastric flora, were found greatly reduced or eliminated in the duodenal flora. For identification, dependence was placed on colony type, blood agar changes, morphology, and particularly fermentations. Among the 25 cases in 7 instances, the streptococci of the duodenum were entirely different from the gastric types, in 16 cases the differences were marked but not complete, and in only 2 cases were the streptococcal types exactly the same. In view of the fact that in most instances there were from 75 to 90 per cent fewer viable streptococci in the duodenal than the gastric content, and that on the other hand the floras of the mouth and gastric content were practically identical in the numbers and types of streptococci, the findings indicate, first, that the duodenal specimens were not seriously contaminated because of the technic employed, and second, that even in the absence of gastric acidity with its inherent bactericidal action and its reported activating influence on the autodisinfecting mechanism of the duodeno-jejunal mucous membrane (Rolly and Liebermeister,[17] 1905, Arnold and Brody,[8] 1926), the conditions in the duodenum still present a fairly effective barrier to the majority of buccal streptococci.

In Table I, the surviving oral streptococcal types are in italics. Only two types frequent in the mouth seemed to survive passage to the rectum with a fair degree of regularity. One type traced through in 5 of the 11 cases is designated as enterococcus on the basis of colony formation, morphology, and the fermentation of esculin. It differed, however, from the typical enterococcus in failure to grow in 2 per cent bile broth and in seldom splitting mannitol. The second type was a chain-forming gamma streptococcus of distinctive cultural characteristics. This was traced through in 3 of the 11 cases. Both of these types were raffinose positive. Aside from *L. acidophilus* and yeasts, they appeared to be the only mouth organisms capable of survival. The types constantly eliminated were the long chain alpha and alpha prime streptococci of the mouth, staphylococci, and also beta streptococci in the one case in which they were noted. The total 25 cases showed viable buccal streptococci in the rectal content in about

50 per cent. They were usually, however, greatly outnumbered by typical intestinal enterococci. It may be concluded then, that even with the barrier of gastric acidity removed, other factors selectively operative in the duodenal region and bacterial antibiosis at lower levels serve to prevent the free passage of most oral streptococci through the small and large intestine.

Although without doubt the maintenance of the scanty flora of the upper small intestine is primarily correlated with the recurrent waves of the normal gastric acid secretions as Arnold and Brody state, persistent alkalinity of the duodenal content is apparently not the only factor which determines the presence or absence of a colonic type of flora at that level. In this series of 26 cases, there occurred an upgrowth of the colonic flora in 15 (57.7 per cent), and the same types of *B. coli* were recovered from the stomach and duodenum as for the stool in 80 per cent, but the establishment of this coliform flora was not particularly linked with the greater degrees of duodenal alkalinity. Thus in 10 cases with a coliform flora in the duodenal region there were 5 with a duodenal alkalinity of pH 7.4 to 7.8, whereas among 11 cases in which there was no such evidence of upgrowth of the colonic flora there were 6 with an alkalinity of pH 7.4 to 7.9.

THE CHARACTER OF THE GASTRODUODENAL FLORA IN RELATION TO BLOOD FINDINGS

The most frequent blood picture shown by these achlorhydria cases was a moderate secondary anemia with a color index somewhat below 1.0, a leucopenia and a relative decrease of the polymorphonuclear leucocytes. There were no examples of pernicious anemia or of typical simple achlorhydric or achylic anemia. In simple achylic anemia, the color index is almost always below 0.6, according to the findings of Menlengracht.[18] Rosenthal and Abel,[19] in a series of 43 cases of simple achlorhydric anemia, reported the color index as between 0.5 and 0.7, but in some instances below 0.5. Of our series, in the 19 cases in which blood examinations were made, there were 9 with a red cell count below 4,000,000 and a hemoglobin finding below 70. None of the cases, however, showed the low color index of achlorhydric anemia, the range being between 8.0 and 1.08. In Tables II and III respectively, there have been grouped the 9 cases showing some degree of anemia, and six cases with a practically normal blood picture, together with the bacterial findings for gastric and duodenal content and the *Cl. welchii* counts for the colonic (stool) flora in order to disclose any possible relation between abnormal distribution of bacteria in the gastrointestinal tract, as regards numbers and types, and the blood findings.

Among the 15 cases detailed in these two tables, there were 6, or 40 per cent, showing upgrowth of the colonic flora to the gastroduodenal region as evidenced by the presence of *B. coli* in varying numbers in specimens from this locality. The three cases (7, 9, 24) showing *B. aerogenes* in small numbers were not considered as showing a definite upgrowth. Among these six cases with upgrowth of the colonic flora to the gastroduodenal region, there were four with a moderate degree of anemia and two with a practically normal blood picture. On the other hand, among the nine with no upgrowths of the colonic flora, at least to the duodenum, there were five with a moderate degree of

TABLE II

GASTRODUODENAL FLORAS OF CASES EXHIBITING SOME DEGREE OF ANEMIA

CASE	SEX AND AGE	BLOOD FINDINGS				BACTERIAL FINDINGS			
		RED CELL COUNT	HB	COLOR INDEX	LEUCOCYTES	GASTRIC CONTENT	DUODENAL CONTENT	HEMOLYTIC ORGANISMS IN GASTRODUODENAL REGION	CL. WELCHII IN STOOL. HIGHEST POSITIVE DILUTION
2. D.	F 40	3,600,000	74	0.97	6,400	Streptoc. + + + + B. coli + +	Streptoc. + B. coli + +	None	10^9
8. G.	F 52	3,900,000	70	0.95	4,800	Streptoc. B. coli + + + +	Streptoc. + B. coli + + + + Cl. welchii + +	Cl. welchii	10^9
11. M.	F 34	3,900,000	65	0.83	8,000	Streptoc. + + + + Staph. aur. +	Streptoc. + Staph. albus +	Streptoc. (beta)	10^1
13. S.	M	3,390,000	62	0.91		Streptoc. + + + Staph. aur. +	Streptoc. +	Streptoc. (alpha prime)	10^2
15. B.	F 40	3,600,000	67	1.08	4,800	Streptoc. + + + Staph. aur. + + Cl. welchii +	Streptoc. + Staph. aur. + Cl. welchii +	Streptoc. (alpha prime) Staph. aur. Cl. welchii	10^5
20. W.	F	2,200,000	55	0.80	4,400	Streptoc. + + + B. coli + + + + Cl. welchii +	Streptoc. + B. coli + + + + Cl. welchii +	B. coli Streptoc. (alpha prime) Cl. welchii	10^9
22. S.	F 22	3,800,000	60	0.80	4,400	Streptoc. + B. coli + + + Staph. aur. +	Streptoc. + B. coli + + +	Staph. aur.	10^3
23. M.	M 69	3,680,000	76	1.06	4,800	Streptoc. + + + + Staph. albus +	Streptoc. + + Staph. aur. +	Staph. aur.	10^3
24. D.	F 43	3,500,000	60	0.86	2,800	Streptoc. + + + + B. aerogenes + Staph. aur. +	Streptoc. + + + B. aerogenes + Staph. aur. +	Staph. aur.	10^7

TABLE III

GASTRODUODENAL FLORAS OF CASES EXHIBITING NO ANEMIA

CASE	SEX AND AGE	BLOOD FINDINGS				BACTERIAL FINDINGS			
		RED CELL COUNT	HB	COLOR INDEX	LEUCOCYTES	GASTRIC CONTENT	DUODENAL CONTENT	HEMOLYTIC ORGANISMS IN GASTRODUODENAL REGION	CL. WELCHII IN STOOL. HIGHEST POSITIVE DILUTION
6. W.	F 40	4,400,000	85	0.99	4,000	Streptoc. + + +	Streptoc. + +	None	10^9
7. S.	M 52	4,700,000	85	0.90	3,200	Streptoc. + + + B. aerogenes +	Streptoc. + + B. aerogenes +	None	10^9
9. S.	M 36	4,800,000	88	0.92		Streptoc. + + + B. aerogenes	Streptoc. + B. aerogenes +	None	10^5
12. T.	M 29	4,360,000	84	0.96	4,400	Streptoc. + + +	Streptoc. + +	None	10^2
14. C.	M 43	4,970,000	90	0.90	4,970	Streptoc. + + + B. coli + Cl. welchii +	Streptoc. + B. coli + +	Cl. welchii (gastric)	10^9
26. F.	M 55	4,200,000	80	0.95	6,000	Streptoc. + B. coli +	Streptoc. + B. coli + +	None	10^9

anemia and four practically normal as regards the blood. These findings, accordingly, afford no definite support to the view that the presence of the *B. coli* group in the gastroduodenal region and presumably throughout the small intestine plays a rôle in the etiology of the anemia in question. It should be noted, however, that in the group with no anemia, the *B. coli*, when present, were fewer than in the positive cases in the anemia group. There appears, on the other hand, to be a fairly regular association of hemolytic organisms (streptococci, staphylococci, *B. coli* or *Cl. welchii*) in the gastroduodenal region with the presence of some anemia. Eight of the nine cases in Table II show this correlation, whereas none of the six cases in Table III (with the exception of a few *Cl. welchii* in the gastric region in Case 12) show any hemolytic organisms in the gastroduodenal region. The series, however, is so small and the number and types of hemolytic organisms recovered so varied, that again the findings lend only slight support to the theory of a possible relationship between absorption of bacterial hemolysins from the gastroduodenal region and the anemia exhibited. There was no correlation between the degree of leucopenia with other blood findings, nor with types or numbers of bacteria in the gastroduodenal region. The presence of high counts of *Cl. welchii* (as determined through spore culture) in the stool specimens is also not correlated with the anemia, in fact quite otherwise as there were more high counts associated with a normal red cell and hemoglobin condition than with the subnormal.

In this series 7 of 9 patients showing anemia were females, and 5 of the 6 patients showing no anemia were males. Thus, although the blood findings did not conform to that considered typical for simple achlorhydric anemia, the sex distribution was much the same. Rosenthal and Abel[19] reported 80 per cent cases in females in their series. These women of our series were also in the age periods forty to sixty years, when achlorhydric anemia is most frequent. Rosenthal and Abel conclude that achlorhydric anemia may be regarded as a disturbance of iron metabolism which occurs chiefly in women during the period of sexual maturity. It would seem probable that the more moderate degrees of anemia exhibited by some of our gastric anacidity cases may be similarly explained, but on the other hand, there exists a possibility that absorption of bacterial hemolysins of one type or another from the gastroduodenal region may be a contributing factor. Such a possibility in reference to Addison's (pernicious) anemia was discussed by Knott[13] in a paper published in 1927.

SUMMARY

A bacteriologic survey of the buccal, gastric, duodenal and colonic floras, in a series of adult patients exhibiting gastric anacidity, is reported.

An upgrowth of the colonic flora to the gastric and duodenal levels was observed in 57.7 per cent of the 26 cases, but no correlation appeared to exist between the occurrence of this upgrowth and the degree of alkalinity of the duodenal content.

The flora of the gastric content was practically identical with that of the mouth. A repeated examination of the gastric content after an interval of

from seven to seventy-nine days in 5 cases showed a marked constancy in bacterial numbers and types regardless of whether or not acid treatment had been given.

In 72 per cent of the cases, the duodenal bacterial counts were 50 to 90 per cent lower than the gastric, due chiefly to destruction of streptococci in the duodenal region. The findings thus indicated that even in the absence of gastric acidity the duodenum still presented a fairly effective barrier to certain swallowed buccal streptococci.

In about 50 per cent of the cases, buccal streptococci were recovered from the stool. Only two types frequent in the mouth seemed particularly capable of surviving; one was an enterococcus-like, esculin-positive diplococcus, and the other a chain-forming gamma streptococcus.

There was no evidence that gastric anacidity was particularly conducive to putrefactive conditions in the large intestine, due to spore-bearing anaerobes.

In about 74 per cent of these cases the *Cl. welchii* count for the stools, as determined by spore culture, was unusually high and equal to those heretofore reported for pernicious anemia.

There were no cases of pernicious anemia in this series, but the majority exhibited some degree of secondary anemia; in 9 the red cell count was below 4 million, and the hemoglobin below 7.0. The color index, however, was not typical for achlorhydric anemia. The anemia was markedly associated with the female sex (80 per cent), and in that respect resembled the sex incidence of typical achlorhydric anemia.

Although there was a rather striking association of hemolytic organisms in the gastroduodenal region with the presence of the anemia, not much weight is, at present, attached to the finding.

REFERENCES

1. MacNeal, W. J., and Chace, A. F.: A Contribution to the Bacteriology of the Duodenum, Arch. Int. Med. **12**: 178, 1913.
2. Kendall, A. I., Day, A. A., Walker, A. W., and Haner, R. C.: The Bacteriology and Chemistry of Adult Duodenal Contents, J. Infect. Dis. **40**: 677, 1927.
3. Venables, J. F., and Knott, F. A.: The Investigation of the Duodenal Contents and Bile in Man, Guy's Hosp. Rep. **74**: 245, 1925.
4. Ricen, L., Sears, H. J., and Downing, L. M.: The Duodenal Flora in Achlorhydria, Am. J. M. Sc. **175**: 386, 1928.
5. Van der Reis, V.: Die Bakterien Flora des Dünndarms und des Coecums bei Erwachsenen unter normalen und pathologischen Verhaltnissen, Klin. Wchnschr. **1**: 950, 1922.
6. Goldman, A.: Studies in Intestinal Bacteriology, I, J. Infect. Dis. **34**: 459, 1924.
7. For a recent review of such observations, reference is made to an article by Nauss, R. W., Lake, M., and Torrey, J. C.: A Critical Analysis of the Lyon Bile Drainage Technic as an Aid to Bacteriologic Diagnosis, J. LAB. & CLIN. MED. **17**: 109, 1931.
8. Arnold, L., and Brody, L.: Bacterial Flora and the H-ion Concentration of the Duodenum, J. Infect. Dis. **38**: 249, 1926.
9. Kopeloff, N.: Is the Stomach a Focus of Infection, Am. J. M. Sc. **165**: 120, 1923.
10. Freeman, R. G., and Miller, E. G.: A Note on a Simple Method for Obtaining Uncontaminated Samples of Intestinal Contents From Various Levels With a Duodenal Tube, J. Bact. **9**: 301, 1924.
11. Torrey, J. C.: A Comparatively Simple Technic for the Bacteriologic Study of Fecal Material, J. Infect. Dis. **39**: 351, 1926.
12. Leonard, V., and Freirer, W. A.: Di-Hydranol, Bull. Johns Hopkins Hosp. **48**: 25, 1931.
13. Knott, F. A.: Addison's (Pernicious) Anemia and Sub-Acute Combined Degeneration of the Cord: The Rôle of Achlorhydria and Intestinal Infection, Guy's Hosp. Rep. **77**: 1, 1927.

14. Moensch, L. M., Kahn, M. C., and Torrey, J. C.: Analysis of the Fecal Flora in Thirty-Three Cases of Pernicious Anemia (with particular reference to B. welchii), J. Infect. Dis. **37**: 161, 1925.
15. Nye, R. N.: Investigation Relative to B. Welchii Infection of the Intestinal Tract as the Etiological Factor in Pernicious Anemia, J. Clin. Investigation **4**: 71, 1927.
16. Torrey, J. C., and Schwartz, H. N.: Bacteriology of the Intestine and Blood in Psoriasis, Arch Dermat. & Syph. **26**: 27, 1932.
17. Rolly and Liebermeister: Experimentelle Untersuchungen über die Ursachen der Abtotung von Bakterien im Dünndarm, Deutsches Arch. f. klin. Med. **83**: 413, 1905.
18. Menlengracht, E.: Simple Achylic Anemia, Acta méd. Scandinav. **78**: 387, 1932.
19. Rosenthal, N., and Abel, H. A.: Simple Achlorhydric Anemia, Bull. N. Y. Acad. Med. **9**: 24, 1933.

THE AGE OF SEXUAL MATURITY IN 250 ALBINO FEMALE RATS (*MUS NORVEGICUS ALBICUS*, WISTAR STRAIN)*

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KELLY[1] in 1933 produced a premature establishment of the vaginal orifice in the rat by the intraperitoneal injection of urine from a pregnant woman. Following this observation, the rat has proved to be a very satisfactory animal for the assaying of certain biologic products. The vaginal orifice spontaneously perforates when the rat reaches sexual maturity. Obviously the exact age of sexual maturity for the normal female rat must be established.

In a review of the literature there is a wide variation in the age of sexual maturity in different strains of rats, and too, a variation in the Wistar strain as shown by the following observations: Donaldson[2] in 1915 gave the approximate age of sexual maturity in a group of albino females of the Wistar strain as sixty to seventy days. Long and Evans[3] in 1922 gave the age as thirty-four to one hundred and nine days in a group of 200 female rats which were a cross from an albino female and a gray male. Freudenberger[4] in 1932 made a comparative study of the Wistar and Long-Evans strains of rats and found that the average age of sexual maturity in a group of 86 Wistar females was 46.9 days and a group of 34 Long-Evans females matured sexually at 52.7 days. Evans and Bishop[5] in 1923 recorded the average age of sexual maturity as 46.8 days in a group of 570 females, the strain of which is not specified.

Since the number of albino females of the Wistar strain used in the different studies has been relatively small, a series of 250 females were observed over a period of approximately one year in order to determine the age of sexual maturity.

METHODS AND PROCEDURES

The rats in this colony were obtained from the Wistar Institute approximately four years ago and have been kept in the Medical School Building where the temperature varies from 66 to 76° F. during the winter and 76 to 90° F.

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during the summer. The rats used in this study were bred over a period of one year and the females were segregated so that the litters would be cast in individual cages.

Some of the young were weaned as early as the twenty-first day while others were never weaned. Some of the young females were segregated from the males on the thirtieth day while others were never separated. The weight of some of the rats was recorded at intervals during the period of observation and to this group has been added the weight of other females of the same strain which were used in another experiment.

The diet during the experiment consisted of commercial dog biscuits (Purina Chow).

The animals were usually examined daily in order to determine when the vaginal membranes spontaneously perforated, as this is considered the date at which the female rat normally becomes sexually mature.

RESULTS

Table I gives the month in which the rats were born and the approximate age of sexual maturity of 250 albino females. Table II gives the average weight at intervals up to fifty-nine days and also the average weight of the animals on reaching sexual maturity.

TABLE I

THE MONTH IN WHICH THE 250 ALBINO FEMALES WERE BORN AND THE APPROXIMATE AGE AT WHICH THEY BECAME SEXUALLY MATURE

AGE IN DAYS	JUNE	JULY	AUGUST	SEPTEMBER	OCTOBER	NOVEMBER	DECEMBER	JANUARY	FEBRUARY	MARCH	APRIL	MAY
20-24												
25-29					1							
30-34		2					1					
35-39	1	6		13		11	8	1	8			
40-44	3	13		8	1	35	21	2	25	1	3	
45-49	4	1		3	2	43	4	2		1	1	
50-54		1		2		18						
55-59						3	1					

TABLE II

THE AVERAGE WEIGHT OF ALBINO FEMALES BETWEEN TWENTY AND FIFTY-NINE DAYS OF AGE AND THE AVERAGE WEIGHT AT THE APPROXIMATE DATE OF SEXUAL MATURITY

AGE IN DAYS	NUMBER OF RATS	AVERAGE WEIGHT GM.	NUMBER OF RATS	AVERAGE WEIGHT AT MATURITY GM.
20-24	14	46.5	0	0.0
25-29	62	57.0	1	80.0
30-34	19	70.7	2	83.5
35-39	22	83.9	30	85.5
40-44	31	91.3	56	91.6
45-49	14	81.1	14	81.1
50-54				
55-59	1	87.0	1	87.0

DISCUSSION

From our study, the age at which the albino female of the Wistar strain matures sexually is approximately thirty days earlier than a group of hybrids reported by Long and Evans[3] and only 4.8 days earlier than a small group of Wistar females reported by Freudenberger.[4] There is no seasonal variation in the age of sexual maturity in the animals used in our study. There is a variation in the weight at maturity although there is no correlation between the age and weight.

When sexually mature, the youngest rat was twenty-eight days of age and the oldest fifty-nine days. It is of interest to note that only one rat out of 250 reaches sexual maturity before thirty days of age.

The age at which females mature is not influenced by the presence of males or the time at which they are weaned. Evans and Bishop[6] in 1922 demonstrated the influence of diet on the rate of development of the rat and also showed that an inadequate diet delayed the time of sexual maturity.

SUMMARY AND CONCLUSIONS

The youngest female to become sexually mature in our series was twenty-eight days old and the oldest fifty-nine days. The average age of sexual maturity in 250 albino females in our colony was 42.1 days. The age of sexual maturity is not influenced by weight, age of weaning, presence of males, or the season of the year.

I wish to thank Doctor E. C. Hamblen of the Department of Gynecology and Obstetrics and Mrs. Susan G. Smith of the Department of Physiology for the opportunity of observing their rats in accumulating data for this study.

REFERENCES

1. Kelly, G. L.: Simple Test for Pregnancy Using Immature Female Albino Rats, J. A. M. A. **100:** 1010, 1933.
2. Donaldson, H. H.: The Rat. Mem. Wistar Institute of Anatomy and Biology, No. 6, Philadelphia, 1915.
3. Long, J. A., and Evans, H. M.: Oestrous Cycle in the Rat. Mem. of the University of Southern California, No. 6, 1922.
4. Freudenberger, C. B.: A Comparison of the Wistar Albino and the Long-Evans Hybrid Strain of Norway Rat, Am. J. Anat. **50:** 293, 1932.
5. Evans, H. M., and Bishop, K. S.: On the Relation Between Fertility and Nutrition, J. Metab. Research **1:** 319, 1922.
6. Evans, H. M., and Bishop, K. S.: On the Relation Between Fertility and Nutrition, J. Metab. Research **1:** 335, 1922.

LABORATORY METHODS

A STUDY OF NONSPECIFIC REACTIONS OF CEREBROSPINAL FLUIDS WITH THE BORDET AND RUELENS ANTIGEN IN THE COMPLEMENT FIXATION TEST FOR SYPHILIS*

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NONSPECIFIC fixation of complement by an acetone insoluble antigen with cerebrospinal fluids has been observed from time to time and was reported by Austin[1] in 1933. The reactions followed fixation for four hours in the refrigerator but not in supplementary tests with fixation for one-half hour in the water-bath at 37° C. These findings necessitated a careful study of specimens which gave such reactions before reports could be submitted. This paper records the results of a study of these reactions.

The technic of the test is described fully elsewhere.[2] Two antigens are used, an alcoholic extract of the acetone-insoluble lipoids of calf- or beef-heart tissue prepared by the method of Bordet and Ruelens[3] and a cholesterolized extract of dried ether-extracted beef-heart tissue prepared by a method similar to that of Neymann and Gager.[4] Fixation with the antigens is accomplished in the refrigerator (3 to 6° C.) during a period of four hours. The individual guinea pig serums used for complement are tested the previous day for (a) hemolytic activity with and without the addition of amboceptor, and (b) nonspecific fixability with antigens by refrigerator and water-bath fixation at 37° C. The satisfactory specimens of serums (not more than twenty-four hours old) are pooled and titrated for hemolytic activity on the morning when they are to be used. The cerebrospinal fluids are inactivated for one-half hour at 55° C. and tested in 0.2 and 0.1 c.c. amounts with controls of 0.4 and 0.2 c.c. amounts.

Fixation of complement ranging from partial (±) to complete (4+) was obtained on an occasional day with a considerable percentage of the specimens of spinal fluid tested with the acetone-insoluble antigen. However, in duplicate tests with the cholesterolized antigen, fixation was obtained only with those specimens from patients having a history of syphilis. For this reason, and because similar discrepancies did not occur with the blood serums from the same patients, the cerebrospinal fluids with which the reactions occurred were always retested the following day.

The lack of correlation between such results and the clinical history of the patients, as well as the difficulty of reproducing the results on successive days, suggested the nonspecific nature of the reactions. The following supplementary

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nonspecific fixability test for guinea pig serum was first devised in order to determine whether the complement might be one of the factors involved in the nonspecific reactions.

To 0.2 c.c. of a single cerebrospinal fluid (previously showing no complement fixation) were added 0.1 c.c. of acetone insoluble antigen (diluted 1:50) and 0.1 c.c. of each of the guinea pig serums (diluted 1:20). The tubes were put into the refrigerator (3 to 6° C.) for two or four hours, at the end of which time 0.2 c.c. of sensitized sheep red cells was added. The tests were then put into the water-bath (37.5° C.) for ten minutes. Any sample of complement with which fixation was obtained at the end of ten minutes was discarded.

By means of this test it was found that there were a few guinea pig serums which reacted nonspecifically with the acetone insoluble antigen and cerebrospinal fluid but which did not react nonspecifically in the preliminary tests with the antigen only. Consequently, these guinea pig serums were not detected previously and were, therefore, pooled with the other serums. With such complement, nonspecific reactions would occur with nearly all of the cerebrospinal fluids tested with the acetone insoluble antigen.

The identification and selection of the individual guinea pig complement with which the nonspecific reactions were observed, afforded an opportunity for the further study of the phenomenon. These serums, for convenience, were designated as serum or complement No. 2 in contradistinction to the pool of serums found entirely satisfactory for use as complement (referred to as No. 1).

EXPERIMENTAL STUDY

Experiment I.—To determine the effect on the complement fixation tests with cerebrospinal fluids of employing complement No. 2 alone and complement No. 2 diluted with complement No. 1.

Complement fixation tests were performed on six cerebrospinal fluids with complement No. 2; complement No. 2 diluted unit for unit with complement No. 1; complement No. 2 diluted one unit with three units of complement No. 1; and complement No. 1 alone. The reactions obtained in these tests are given in the following protocol. It is apparent that the nonspecific reactions may occur if complement No. 2 is not sufficiently diluted.

While anticomplementary reactions were not obtained with complement No. 2 in the preliminary test for nonspecific fixability with acetone insoluble antigen alone, reactions were frequently obtained with this complement the following day in the antigen control tests.

PROTOCOL 1

PROTOCOL OF COMPARATIVE COMPLEMENT FIXATION TESTS WITH COMPLEMENT NO. 2

CEREBROSPINAL FLUID NOS.	COMPLEMENT NO. 2 DILUTED TO CONTAIN 2 UNITS IN 0.1 C.C.		COMPLEMENT NO. 2 DILUTED UNIT FOR UNIT WITH COMPLEMENT NO. 1		COMPLEMENT NO. 2 DILUTED 1 UNIT WITH 3 UNITS OF COMPLEMENT NO. 1		COMPLEMENT NO. 1 DILUTED TO CONTAIN 2 UNITS IN 0.1 C.C.	
	ACETONE INSOLUBLE ANTIGEN	CHOLESTEROLIZED ANTIGEN	ACETONE INSOLUBLE ANTIGEN	CHOLESTEROLIZED ANTIGEN	ACETONE INSOLUBLE ANTIGEN	CHOLESTEROLIZED ANTIGEN	ACETONE INSOLUBLE ANTIGEN	CHOLESTEROLIZED ANTIGEN
729	3+	–	–	–	–	–	–	–
800	3+	–	±	–	–	–	–	–
830*	4+	4+	4+	4+	4+	4+	4+	4+
831	2+	–	±	–	–	–	–	–
832	3+	–	–	–	–	–	–	–
846	3+	–	±	–	–	–	–	–

*History of Syphilis.

Thus, by excluding the No. 2 serums from specimens of pooled complement, the nonspecific reactions with the cerebrospinal fluids are not only avoided, but the number of anticomplementary reactions in the routine antigen controls such as were reported by Plaut[5] and Austin[1] is also reduced.

Since similar irregular reactions (Table I) were not observed in the complement fixation tests with blood serums, the effect of adding small amounts (0.03 or 0.05 c.c.) of inactivated normal human serum was studied, the serum being added to the tube before the cerebrospinal fluid. It was found, as in Plaut's[5] experiments, that the serum protected against nonspecific reactions with cerebrospinal fluids.

The observation was made at this time that with the same complement and cerebrospinal fluids nonspecific reactions did not occur with all preparations of acetone insoluble antigen, a fact which indicated that the complement was only a secondary factor in the occurrence of the reactions. Since the lipoid (cephalin) content of each antigen probably varied, it was suggested that the nonspecific reactions might be correlated with the cephalin content of the antigen. The following experiments were carried out.

Experiment II.—To determine the effect of varying the cephalin content of an ether-extracted acetone insoluble antigen on complement fixation of cerebrospinal fluids with complement 2.

In the preparation of antigens by the method of Bordet and Ruelens, the tissue does not receive a preliminary extraction with ether and therefore the extracts probably contain considerable cephalin. The antigen B XIV-D,[6] employed in this experiment, was prepared from heart tissue which had been subjected to preliminary extraction with both acetone and ether. The total mixed lecithin and cephalin in this antigen, calculated from lipoid phosphorus, after the preliminary extraction with ether was reduced from 5.371 to 3.739 mg. per c.c.

Amounts of 0.2, 0.5, and 1 c.c. of a 0.1 per cent ether solution of purified cephalin[7] were added, respectively, to three watch glasses and evaporated by means of an electric fan. The dried material was streaked in 1 c.c. of 95 per cent alcohol, then to each of the alcoholic suspensions was added 0.6 c.c. of the antigen B XIV-D and the contents of each watch glass were mixed well and evaporated. Finally, each of the dried residues was emulsified in 1.5 c.c. of distilled water and 0.5 c.c. of each emulsion made up to a final dilution of 1:75 with physiologic salt solution, the optimal dilution of the antigen. One-tenth cubic centimeter (the antigenic dose) of the diluted antigens contained, respectively, 0.00044, 0.0011, and 0.0022 mg. of additional cephalin.

Comparative complement fixation tests were then carried out on seven cerebrospinal fluids with complement 2 and the following antigens: the ether-extracted acetone insoluble antigen B XIV-D; the three samples of this antigen to which cephalin had been added, as described previously; and two acetone insoluble antigens (B XI and B XIV-C) prepared from tissue which had not been extracted with ether. The former, B XI, was prepared by the method of Bordet and Ruelens; the latter, B XIV-C, by a modification of this method which included a more thorough preliminary extraction with acetone and final extraction with absolute, rather than 95-per-cent alcohol. The total calculated phospholipids in this antigen were 4.894 mg. per c.c.

Results: Nonspecific reactions were obtained with all of the cerebrospinal fluids and the acetone insoluble antigen B XI and with six out of seven of the specimens with acetone insoluble antigen B XIV-C, while no nonspecific reactions were obtained in any instance with the ether-extracted acetone insoluble antigen B XIV-D (the latter results agreeing with those obtained in the routine tests). When cephalin was added to the antigen B XIV-D so that an antigenic dose of the diluted antigen contained no more than 0.00044 mg. of additional cephalin, the results with the antigen were unaltered. When, however, the cephalin content was increased to 0.0011 mg., nonspecific reactions occurred with four of the seven cerebrospinal fluids; and by further increasing the amount of cephalin (0.0022 mg.), a still larger number of nonspecific reactions was obtained, these results agreeing more closely with those obtained with the antigen B XI (Table I).

In short, although complement 2 was employed, the ether-extracted acetone insoluble antigen B XIV-D did not give rise to nonspecific reactions with cerebrospinal fluids until after the addition of cephalin as did the antigens B XI and B XIV-C which were prepared according to the method of Bordet and Ruelens.

TABLE I

THE EFFECT OF VARYING THE CEPHALIN CONTENT OF AN ACETONE INSOLUBLE ANTIGEN ON COMPLEMENT FIXATION OF CEREBROSPINAL FLUIDS WITH COMPLEMENT 2

CEREBRO-SPINAL FLUID NOS.	ACETONE INSOLUBLE ANTIGEN B XI (DIL. 1:75)	ACETONE INSOLUBLE ANTIGEN B XIV-C (DIL. 1:75)	ETHER-EXTRACTED ACETONE INSOLUBLE ANTIGEN B XIV-D (DIL. 1:75)	B XIV-D ANTIGEN PLUS CEPHALIN		
				0.0004 MG. IN 0.1 C.C. OF DILUTED ANTIGEN	0.0011 MG. IN 0.1 C.C. OF DILUTED ANTIGEN	0.0022 MG. IN 0.1 C.C. OF DILUTED ANTIGEN
29998	+	–	–	–	–	–
30907	3+	2+	–	–	±	2+
28011	3+	±	–	–	±	3+
20237	3+	+	–	–	±	3+
35167	3+	+	–	–	±	+
30286	3+	±	–	–	–	±
30706	3+	+	–	–	–	±

Experiment III.—To determine the effect of varying the cephalin content of the ether-extracted acetone insoluble antigen on complement fixation of cerebrospinal fluids with different specimens of complement.

Complement fixation tests were performed on cerebrospinal fluid (previously showing no fixation) with six different samples of complement and the following antigens: B XIV-D, two portions of B XIV-D which contained, respectively, 0.0022 and 0.0044 mg. of additional cephalin per 0.1 c.c. of diluted antigen, and the acetone insoluble antigen B XI.

Results: Table II shows that the cephalin content of the acetone insoluble antigen apparently had a direct influence on the occurrence of nonspecific reactions with cerebrospinal fluid. No nonspecific reactions occurred in the complement fixation tests on cerebrospinal fluid with the ether-extracted acetone insoluble antigen until after the addition of cephalin (0.0022

TABLE II

THE EFFECT OF INCREASING THE CEPHALIN CONTENT OF AN ACETONE INSOLUBLE ANTIGEN ON COMPLEMENT FIXATION OF CEREBROSPINAL FLUID WITH DIFFERENT SPECIMENS OF COMPLEMENT

COMPLEMENT	ACETONE INSOLUBLE ANTIGEN B XI	ACETONE INSOLUBLE ANTIGEN B XIV-D	B XIV-D ANTIGEN PLUS CEPHALIN	
			0.0022 MG. IN 0.1 C.C. OF DILUTED ANTIGEN	0.0044 MG. IN 0.1 C.C. OF DILUTED ANTIGEN
1	±	–	3+	4+
2	–	–	3+	4+
3	+	–	2+	3+
4	–	–	±	3+
5	3+	–	2+	4+
6	–	–	3+	4+

or 0.0044 mg. to 0.1 c.c. of diluted antigen). Nonspecific cerebrospinal fluid reactions were obtained with the acetone insoluble antigen B XI and three out of the six samples of complement. Antigen B XI was not subjected to a preliminary extraction with ether and undoubtedly contained a comparatively large amount of cephalin, judging from the calculated mixed cephalin and lecithin per cubic centimeter of antigen B XIV, similarly prepared.

The ether-extracted antigen was not anticomplementary while the antigen B XI showed anticomplementary properties with two of the six complements. After addition of the cephalin

(0.0022 or 0.0044 mg. per antigenic dose) to antigen B XIV-D, nonspecific properties were revealed in the antigen. The sample to which the larger amount of cephalin (0.0044 mg.) had been added was markedly anticomplementary, all six of the samples of complement being fixed nonspecifically. The anticomplementary action of that portion of the antigen to which 0.0022 mg. of cephalin had been added was slightly greater than that of the antigen B XI. In further experiments we found that when amounts of cephalin of from 0.0011 to 0.00177 mg. (per antigenic dose) were added to the ether-extracted antigen, the anticomplementary properties of the antigen paralleled those of the B XI antigen. When amounts of cephalin of less than 0.0011 mg. (per antigenic dose) were added fewer anticomplementary reactions were observed with this antigen than with the antigens B XI or B XIV-C.

Maltaner,[8] and Wadsworth, Maltaner, and Maltaner[9, 10] have discussed the anticomplementary property of cephalin and its lack of antigenic action with syphilitic serum. The latter authors[10] have also shown that within certain limits the anticomplementary property of cephalin was inhibited by normal or syphilitic serums. In our experiment 0.1 c.c. of the suspension of cephalin containing 0.0022 or 0.0044 mg. per 0.1 c.c. was not anticomplementary, nor was 0.2 c.c. or 0.4 c.c. of the lighter suspension anticomplementary. We found, however, that although the weaker suspension did not react nonspecifically with complement alone, when 0.1 c.c. was employed in complement fixation tests with cerebrospinal fluid, marked anticomplementary reactions occurred.

In a preliminary study of complement 2, tests were conducted to determine whether the nonspecific reactions might be correlated with the residual fibrinogen in the complement. Removal of the fibrinogen was accomplished by following the technic described by Maltaner and Johnston[11] for producing secondary clots in calf serum. The nonspecific reactions were not prevented, but the fact that the titer of the complement was considerably reduced by secondary clotting is of interest.

SUMMARY AND CONCLUSION

1. These experiments suggest a correlation between the nonspecific reactions in the complement fixation test for syphilis with cerebrospinal fluids and the cephalin content of the acetone insoluble antigens prepared by the method of Bordet and Ruelens. (Nonspecific reactions were not obtained with the acetone insoluble antigen prepared from heart tissue which had been subjected to preliminary extraction with both acetone and ether.)

2. The property of nonspecific fixability of certain guinea pig serums by acetone insoluble antigen and cerebrospinal fluid is a secondary factor in causing nonspecific reactions in complement fixation tests for syphilis on cerebrospinal fluids. A test is described for detecting such serums.

3. The Bordet-Ruelens antigen is unreliable in the complement fixation test for syphilis on cerebrospinal fluid unless there has been preliminary extraction with ether as well as with acetone, or unless the guinea pig serums with which nonspecific reactions may occur are eliminated from the pool of complement used in the tests.

REFERENCES

1. Austin, H. A.: The Nonspecific Fixability of Guinea-pig Serum With Bordet's Antigen, J. Lab. & Clin. Med. **18**: 389, 1933.
2. Wadsworth, A. B.: Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, Baltimore, 1927, Williams & Wilkins Co., p. 177.
3. Bordet, J., and Ruelens, G.: L'Antigene syphilitique de l'Institut Pasteur de Bruxelles, Comp. rend. Soc. de biol. **82**: 880, 1919.
4. Neymann, C. A., and Gager, L. T.: A New Method for Making Wassermann Antigens From Normal Heart Tissue, J. Immunol. **2**: 573, 1917.

5. Plaut, F.: Über die Anwendung der Kältebindung bei der Wassermannschen Reaktion des Liquor cerebrospinalis, Ztschr. f. d. ges. Neurol. u. Psychiat. **138**: 169, 1932.
6. Annual Report of the Division of Laboratories and Research, 1933, p. 19. (The preparation of antigens for complement-fixation tests for syphilis—Frank Maltaner.)
7. Maltaner, F.: A Note on the Preparation of Cephalin, J. Am. Chem. Soc. **53**: 4019, 1931.
8. Maltaner, E.: The Absence of Antigenic Activity in the Complement-Fixation Test of the Purified Lipoids, Cephalin and Lecithin (Abstract), Am. J. Path. **7**: 537, 1931.
9. Wadsworth, A., Maltaner, F., and Maltaner, E.: Studies on the Coagulation of the Blood, Trans. Assn. Am. physicians **46**: 296, 1931.
10. Wadsworth, A., Maltaner, E., and Maltaner, F.: A Study of the Antigenic Properties of Lecithin and Cephalin, J. Immunol. **26**: 25, 1934.
11. Maltaner, F., and Johnston, E.: Observations Upon the Conglutination Phenomenon, J. Immunol. **6**: 349, 1921.

IDE TEST—THE NEW COLOR TEST FOR SYPHILIS*

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THIRTY years ago Wassermann introduced into the medical world his world-famed test for syphilis. The principle of this test is based on the complement fixation or the complement deviation reaction originally brought forth by medical celebrities such as Bordet, Gengou and Ehrlich, and its use is not limited only to syphilis but is widely applied in other diseases, among which may be mentioned tuberculosis, gonorrhea, and typhoid fever. The test, however, as is well known, is a biologic one requiring the antigen and the amboceptor obtained from the immunized serum and the red corpuscles of certain animals, and the complement supplied by guinea pig's serum. Consequently the Wassermann test is a very elaborate and complicated procedure taxing an enormous amount of energy and accuracy from those who are engaged in its performance. In order to overcome this difficulty its substitute in the form of precipitation tests has been introduced of which the most well known are those devised by Meinicke, Sachs-Georgi, Kahn, Murata, etc. Some workers, however, are inclined to think that these tests are perhaps not as specific and accurate as the Wassermann reaction.

The new test which the authors wish to publish here has special advantages, namely, with only one drop of blood and a simple technic, the result can be read clearly by the coloring under the microscope. It can be made in a few minutes' time in one's own examining room. The accuracy of this test compares very well with those of Wassermann, Meinicke, Sachs-Georgi, Kahn, Murata, etc., and it can also be performed with blood serum, spinal fluid or serous exudate obtained from vesicles.

Production of the Antigen or the Test Fluid.—For this purpose beef heart is used. After removing the fat, fibrous tissue and blood vessels, the heart

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muscle is ground several times by means of an ordinary meat grinder. The ground muscle is then treated by 95 per cent alcohol. A suitable container, with tightly fitting cork stopper, containing the muscle and the alcohol in the proportion of 20 gm. to 100 c.c. is placed in a hot water-bath of temperature 60° C. and kept there, at least, for seven weeks. During this period the contents should be shaken regularly, once in the morning and again in the evening. At the end of seven weeks or more the contents are cooled, filtered, and kept in a dark place as stock solution. (Note: If necessary the contents may be used after keeping them in the water-bath for four weeks. The best result, however, is obtained by keeping them in the bath at least for seven weeks. The cork stopper used must be of good length so as to fit well down into the mouth of the container. In addition it should be tied down to the container itself. This is necessary, because keeping the container in a hot water-bath for such a long period of time tends to cause rotting of the stopper and the subsequent leaking of the contents.)

To 100 c.c. of the stock solution prepared as above, 0.2 gm. of cholesterin is added and dissolved by shaking in a warm water-bath (56° C.) for about ten minutes. Let us call this Fluid 1.

With another portion of the stock solution, a 5 per cent solution of gum benzoin is made. Let this be Fluid 2.

Now prepare a 1 per cent alcoholic solution of crystal violet. Let us call this Fluid 3.

Similarly, prepare a 1 per cent alcoholic solution of Azure II. This we call Fluid 4.

To 100 c.c. of Fluid 1 are added 5 c.c. of Fluid 2 and are mixed thoroughly. One cubic centimeter of Fluid 3 and 1 c.c. of Fluid 4 are mixed well together, and 1 c.c. of this mixture is added to the No. 1 and No. 2 mixture. The purplish blue fluid thus obtained is the finished test fluid or the "antigen" and is now ready to be used. (Note: The amount of the dyes used may be slightly more or less than 1.2 c.c. according to their effectiveness. It is absolutely necessary that the dyes and cholesterin used are of Merck products.)

Procedure of the Test.—1. Get a drop of blood from lobe of patient's ear or fingertip with a blood drawing pipette. (Blood drawn from a vein will also do.) The amount of blood drop will measure about 0.03 c.c. (Less than this amount should not be used.) Place this drop on the concavity of a hollow slide, especially made for this purpose (see below). With a saline solution dropping pipette, put on this one drop (0.05 c.c.) 3.5 per cent saline solution, stir it with corner of ordinary object glass, and at the same time, spread it out over the whole surface of the depression. If this spreading out is neglected, when shaking process comes, the fluid will not mix well with diluted antigen.

2. Next take 0.2 c.c. of Ide antigen into small test tube, and to this add 0.6 c.c. of 2.5 per cent sodium chloride solution. The tube is then shaken about 30 times, mixing 2 fluids thoroughly. This is the diluted Ide antigen.

3. Add one drop (about 0.03 c.c.) of this diluted Ide antigen to blood and saline mixture described in paragraph 1. Then the hollow slide must be placed flat on the desk and should be shaken rather vigorously for three or four minutes, forward and backward as well as sideways, so that the fluids in the depres-

sion are seen to whirl about. In order to get this whirling movement of the fluids one must first spread the blood and saline mixture over the whole surface of the depression. After this shaking, examine it under microscope (about 50 magnification). One must choose the thin layer of the fluids toward the periphery of the depression with bright light for clearest exposition.

Positive reaction is evidenced by the appearance of beautifully purplish blue colored clumps among the red blood corpuscles in the field, while, if the reaction is negative, no such changes occur and the red blood cells alone can be recognized. If the reaction is very strongly positive, one is able to see with the naked eye, even in the course of shaking, the appearance of purplish blue masses. However, when the reaction is only weakly or doubtfully positive, one sees the tiny purplish blue bodies only under the microscope.

Method of Applying Ide Test to Blood Serum, Spinal Fluid, and Exudate Obtained From Vesicles.—Place 0.03 c.c. of the above mentioned material on the hollow slide and spread out over entire surface of depression. Then put one drop of diluted Ide antigen directly on the materials and shake hollow slide for about five minutes in same manner as described elsewhere. Then make microscopic examination. (On this material it is not necessary to put 3.5 per cent saline solution.) Microscope examination will show about the same thing as blood examination, with slightly thinner color of purplish masses.

Points to Be Watched in Performing Test.—

1. Materials used in this test:

    1. Patient's blood.
    2. Ide antigen.
    3. 3.5% sodium chloride for diluting blood.
    4. 2.5% sodium chloride solution for diluting Ide antigen.

2. In performing this test, one should obtain a specially made hollow glass having 2 or 3 depressions with a diameter of about 2 cm., a depth of about 2 mm., and a cover glass large enough to cover the whole slide.

3. The proportion of the Ide antigen to that of the saline should always be 1 to 3 and at least 0.2 c.c. of the Ide antigen should be used in making the dilution. It must be remembered that the diluted Ide antigen should be used within thirty minutes of its dilution, otherwise a coarse precipitate may form. Originally there are many greenish particles large and small in the diluted Ide antigen. These particles disappear when mixed with negative blood or serum. However, when mixed with positive blood or serum, these particles not only do not disappear but form a big purplish blue lump.

4. The result of the test should be read within fifteen minutes after shaking the slides, otherwise dyes themselves may be seen to float on the surface and interfere with accurate reading, especially in cases of weakly or doubtfully positive reactions.

5. After shaking the slide one must place a glass cover over the slide in order to prevent evaporation. One should try to examine the result through the glass cover.

6. As a rule, at a low temperature, between the months of October and May, cholesterin in the Ide antigen precipitates out. Therefore, it is well to keep

Fig. 1.

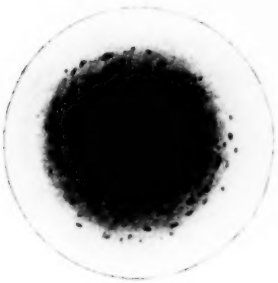

Fig. 2.

Fig. 3.

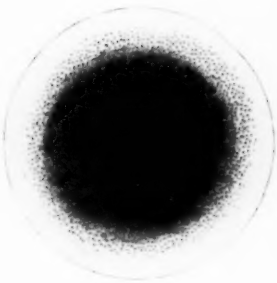

Fig. 4.

Plate I

Fig. 1.—Superstrong positive. Violet blue masses seen with naked eye.
Fig. 2.—Strong positive. Violet blue masses seen under the microscope (×50).
Fig. 3.—Weak positive. Small violet blue granules seen under microscope (×50).
Fig. 4.—Negative.

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the Ide antigen in an incubator or, if that is not available, in a small box equipped with an electric bulb (5 watts). When the cholesterin precipitates out, it sinks down to the bottom of bottle in the form of small white needles. If the cholesterin has already precipitated out, it should be redissolved before using the Ide antigen by shaking the bottle for five minutes or more in a water-bath at 50° to 60° C.

7. With the blood obtained from long-treated patients, this test may yield on a very few occasions a negative reaction where the reaction may be positive by the Wassermann test. In such cases, the test should be done after heating the blood for twenty minutes in a warm water-bath, about 56° C., and then cooling it. The technic is as follows: 4 or 5 drops of the patient's blood are obtained in a small test tube and are stirred for three or four minutes in order to defibrinate the blood. The defibrinated blood is then placed in the above mentioned warm water-bath. The blood thus treated usually gives a positive reaction.

8. Sometimes in some part of the field a few bluish particles resulting from dirt coming from the outside can be seen. But this can be differentiated from positive reaction, because in positive reaction, numerous masses can be seen everywhere in the field.

9. The dropper used should be gauged so as to deliver a drop of 3.5 per cent sodium chloride solution approximately measuring 0.05 c.c. Such a dropper will deliver a drop of the antigen approximately measuring 0.03 c.c. This difference is due to the difference of specific gravity. These droppers can be easily made with pipette gauge.

10. Before puncturing, the alcohol on the ear lobe should be dried, otherwise blood will spread out over the alcohol area.

11. If any precipitate forms in the bottle of the blood diluting saline solution, it should be freshly made.

Comparison of the Wassermann and Ide Tests in 1,262 Cases

Wassermann			Ide	
3-plus reactions	113	119 strongly positive	100	116 strongly positive
2-plus reactions	6		16	
1-plus reactions	10		3	
Plus-minus reactions	7		3	
Negative reactions	1126		1140	

From the above results, it can be seen that of the 119 cases giving the strongly positive Wassermann reactions, 116 gave strongly positive Ide reactions. Apparently 3 cases failed to agree. Of the 17 cases giving weakly or doubtfully positive Wassermanns, 6 gave weakly or doubtfully positive Ide. Eleven failed to react. It is to be noted that none of the 1,262 cases yielding negative Wassermann reactions gave positive Ide reactions.

Through this paper S. Ide wishes to thank Professor Albert M. Barrett of the University of Michigan who showed him great kindness during his years in the United States. Thanks are also due to Professor David M. Cowie of the Pediatrics Department of the University of Michigan and Dr. Frank Smithies of Chicago. Also George J. Smith, formerly assistant in the Serological Laboratory of the University of Michigan.

We wish to thank the Honorable Professor Kinnosuke Miura of the Imperial University of Tokyo, now Director of Doai Memorial Hospital, by whose generosity we are able to pursue our studies.

REFERENCES

1. Cheviak: Arch. med. inf. Hosp. Garcia **1:** 3, 125 and 137, 1932.
2. Dahr, Peter: Deutsche med. Wchnschr. **45:** 1934.
3. Dahr, Peter: Deutsche med. Wchnschr. **3:** 94, 1934.

The authors of this paper, having sent a supply of antigen and sets to Professor A. M. Barrett of the University of Michigan, have asked Professor Barrett to kindly furnish antigen and set, free of charge, upon request, to those who wish to try this method.

A CONVENIENT FOOD CUP FOR RATS*

WILLIS D. GALLUP, M.S., STILLWATER, OKLA.

A RAT-FEEDING device for use with compressed food pellets has been described by Baumberger and Allen in a recent issue of this JOURNAL.[1] In a comparison of the merits of compressed food with those of a ground food mixture, the authors point out that the latter is not only inconvenient to handle but is also subject to much waste through scattering and contamination by the animals. The use of compressed pellets with the device described overcomes these objectionable features to a degree.

A ground food mixture prepared in the laboratory has an advantage, however, in that its composition may be varied at will and such supplements

Fig. 1.

included in the mixture as might be desired under different dietary regimen. To prevent scattering of the food, a special type of food cup has been devised and found to be very satisfactory.

The food container is a half-pint round mason jar. A piece of hardware cloth is rolled into the form of a hollow cylinder 2½ inches high and 1½ inches in diameter. The first two cross wires at the top are cut in 6 places to permit the edge of the cylinder to be bent outward perpendicular to the side. This edge is rounded with a pair of tin shears and the cylinder suspended in the jar with its bent edge resting on the top edge of the jar. A hole 1 inch in

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diameter is cut in the lid of the jar and the rough edge crimped under with a pair of pliers. The lid, when screwed in place, secures the wire mesh cylinder.

The purpose of the cylinder which forms a false side of the container is to prevent the rat from drawing large amounts of food up the side of the jar and throwing it out with its fore paws while eating. Lids with a larger hole may be used for very large rats.

These food containers are particularly suitable for use with false bottom cages. The base of the jar is sufficiently large to prevent easy tipping although occasionally large animals persist in tipping the jar on its side. Whenever this happens, the jar is placed in an ordinary tin drinking cup. The amount of food in the jar is always visible. The round container is easily cleaned, and since its parts are of uniform standard size, they are interchangeable and easily replaced.

REFERENCE

1. Baumberger, J. P., and Allen, S.: A New Rat Feeding Device, J. LAB. & CLIN. MED. 20: 970, 1935.

THE INTERFERENCE OF NITRITES IN THE DETECTION AND ESTIMATION OF UROBILINOGEN IN URINE*

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THE purpose of this communication is to draw attention to the interference of nitrites with Ehrlich's dimethylamidobenzaldehyde reaction for the detection and estimation of urobilinogen in urine.

When Ehrlich's reagent is added to urine, the hydrochloric acid, which the reagent contains, liberates nitrous acid from nitrites in the urine. Nitrous acid, being a powerful oxidizing agent, oxidizes the urobilinogen and thus prevents the formation of the colored compound with dimethylamidobenzaldehyde, by means of which the urobilinogen is detected and measured. Normally, *freshly voided* urine contains very faint traces only of nitrites. As will presently be shown, however, with infection of the urinary tract with bacteria which can reduce nitrates to nitrites (*B. coli*, etc.), the amounts of nitrite in the urine may be sufficient to seriously interfere with Ehrlich's reaction.

The observation that nitrites interfere with Ehrlich's reaction is not new. Hoesch[1, 2] first observed this phenomenon. Römcke and Schrumpf[3, 4] and Schrumpf[5] observed that, in decomposing urines, as nitrites were formed, Ehrlich's reaction became weaker or negative, but attributed these findings to oxidation of urobilinogen from liberation of oxygen during the reduction of nitrates to nitrites. The fact, noted by Hoesch,[1, 2] that addition of pure sodium nitrite to urine interferes with Ehrlich's reaction was apparently overlooked.

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As far as could be ascertained from the literature, the smallest amount of nitrites which may interfere with detection and estimation of urobilinogen in urine with this reagent has never been accurately determined. Nor has a study been made of the occurrence of such minimal quantities in *freshly voided* urines.

Method of Investigation.—Studies were made of the nitrite contents of urines of individuals with and without infections of the urinary tract.* The Griess-Ilosva test for nitrites was used. The reagent was prepared as described by Treadwell and Hall.[6] One cubic centimeter of reagent was added to 10 c.c. of the solution tested, and the mixture was allowed to stand ten minutes to permit maximum color development. The test was applied to the clear colorless filtrates which were obtained by treating the urines with blood charcoal.† The colors produced were then compared with standards consisting of mixtures of the reagent with aqueous solutions of sodium nitrite of known nitrite content. The concentrations of the standards ranged between one part of sodium nitrite in 100 million parts of water and 1 part in 5 million. When the nitrite content of the urine was found to be greater than 1 in 5 million, the test was repeated with suitable dilution of the urine filtrate. All tests were performed no later than thirty minutes after the urines were collected.

Sensitivity of Reaction.—The minimum concentration of sodium nitrite which could be detected by the above procedure was, with aqueous solutions 1 in 100 million, and with urines 1 in 20 million.

Nitrite Content of Freshly Voided Urines.—With no infection of the urinary tract, the colors obtained corresponded to those given by aqueous solutions, containing one part of sodium nitrite in 15 to 20 million parts of water. With infection, concentrations of nitrite of 1 in 25,000 to 1 in 100,000 were frequent; in one instance it was as high as 1 in 15,000.

Influence of Nitrites on Ehrlich's Reaction for Urobilinogen.—In order to determine the effect of various concentrations of nitrite on the urobilinogen reaction, Ehrlich's reagent was added to urines with a nitrite concentration of about 1 in 20 million, and to the same urines which contained known amounts of added sodium nitrite. The minimum concentration of sodium nitrite which interfered perceptibly with the reaction was found to be about 1 in 500,000; concentrations of 1 in 100,000 invariably produced some interference and concentrations of 1 in 50,000 to 1 in 25,000 inhibited the reaction almost completely even with urines of high urobilinogen content.

From the above results, the limitations of the test are obvious. It should be observed that the concentration of nitrite usually present in urines from patients without infection of the urinary tract, that is about 1 in 15 to 20 million, is well below the minimum concentration which interferes with Ehrlich's reaction. On the other hand, urines from patients with infection may contain

*Increased amounts of nitrite in the urine have been reported in conditions other than infection of the urinary tract (enterogenous cyanosis, etc.), but such conditions are rare.

†I have found that addition of the reagent to charcoal filtrates, instead of to untreated urine, greatly increases the sensitivity of the test and also permits approximately quantitative analysis by comparison of the color obtained with standards of known nitrite content. Recovery experiments showed that blood charcoal did not interfere with quantitative analysis.

sufficient nitrite to seriously interfere with the reaction and, in some cases, to even almost completely inhibit it when the actual urobilinogen content of the urine is high.

The Green Color Reaction.—It is well known that, occasionally, on addition of Ehrlich's reagent to urine which contains bile, the reaction mixture rapidly becomes dark green. Hoesch[1] has shown that this is due to oxidation of the bilirubin by the nitrous acid which is liberated by the action of the hydrochloric acid of the reagent on the nitrites in the urine. In one of our patients in whom the urine was markedly pigmented with bile and in which this green color reaction was observed, the nitrite content of the urine was found to be ranging between 1 in 25,000 and 1 in 75,000. This was a case of *B. coli* infection of the urinary tract. Experiments with bile-containing urines, to which sodium nitrite was added, showed that the minimum concentrations of nitrite required to produce the green color reaction ranged between 1 in 500,000 and 1 in 25,000.

SUMMARY

Attention is drawn to the interference of nitrites in urine with Ehrlich's reaction for the detection and estimation of urobilinogen in urine. The minimum concentration of sodium nitrite which interfered perceptibly with the reaction was found to be about 1 part of nitrite in 500,000 parts of urine. Concentrations of 1 in 50,000 to 1 in 25,000 were found to inhibit almost completely the reaction even in urines of high urobilinogen content.

With no infection of the urinary tract, the amount of nitrite usually present is not sufficient to interfere with the reaction. With infection with organisms which can produce nitrites, the test is of no value.

Attention is drawn to the green color which may be produced on addition of Ehrlich's reagent to urines which contain bile and nitrites.

REFERENCES

1. Hoesch, K.: Über die sogenannte grüne Benzaldehydreaktion im Bilirubinharn, Klin. Wchnschr. **1**: 2034, 1922.
2. Hoesch, K.: Zur Urobilinogenurie, Biochem. Ztschr. **167**: 107, 1926.
3. Römcke, O., and Schrumpf, A.: Einige Urobilin- und Urobilinogenstudien, Ztschr. f. d. ges. exper. Med. **74**: 458, 1930.
4. Römcke, O., and Schrumpf, A.: Studien über Urobilin und Urobilinogen, Norsk. Mag. f. Laegevidensk. **92**: 514, 1931; abstracted in Ber. ü. d. ges. Physiol. u. exper. Pharmakol. **62**: 602, 1931.
5. Schrumpf, A.: Einige weitere Urobilinogen und Urobilinstudien, Ztschr. f. d. ges. exper. Med. **79**: 564, 1931.
6. Treadwell, F. P., and Hall, W. T.: Analytical Chemistry, New York, 1921, John Wiley and Sons **1**: 352.

THE MICRODETERMINATION OF SODIUM BY THE URANYL ZINC ACETATE METHOD AND THE TITRATION OF URANIUM WITH CADMIUM AS THE REDUCTANT*

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SINCE the introduction of methods employing the triple acetate of uranyl sodium and zinc or magnesium for the gravimetric determination of sodium (Blanchetière, Barber and Kolthoff, Caley and Foulk), numerous modifications of the original procedure have appeared in the literature on microanalysis. Usually sodium has been indirectly determined by estimating the amount of uranium either colorimetrically with $K_4Fe(CN)_6$ or by titration with a solution of $KMnO_4$. In our work on the determination of the asn constituents of the genital tract, a micromethod for sodium was required which would be simple, rapid, and accurate. The colorimetric method was first used, but due to the small amount of the material available and the uncertainty of the sodium content of the tissues, it was abandoned in favor of a volumetric method which was devised to meet our requirements.

The triple acetate dissolved in the uranyl zinc acetate reagent was precipitated by the addition of alcohol (McCance and Shipp); this is essential for the rapid and complete precipitation of the triple salt. The quantity of alcohol, whether 20 or 10 per cent (Salit, Marenzi and Gerschman) was immaterial; but a blank determination of the reagents was always necessary. Potassium in an amount proportional to that found in smooth or striated muscle apparently was not precipitated by the alcoholic reagent. In titration the hexavalent uranium was reduced by cadmium to the quadrivalent state, and the latter was oxidized by $KMnO_4$. The details of the entire process are as follows:

EXPERIMENTAL

Reagents—

1. Uranyl zinc acetate reagent. (A) 10 gm. of uranyl acetate are dissolved in 50 c.c. of boiling water containing 2 c.c. of glacial acetic acid. (B) 30 gm. of zinc acetate are dissolved in 50 c.c. of boiling water containing 1 c.c. of glacial acetic acid. The hot solutions, A and B, are mixed and heated to boiling. The solution is then allowed to stand twenty-four hours and is filtered before use.

2. Glacial acetic acid saturated with sodium zinc uranium acetate as prepared by Salit, centrifuged just prior to use.

3. Absolute ether.

4. Cadmium pencil (a cadmium stick sharpened at one end to fit the tip of a centrifuge tube).

5. Cadmium powder, prepared by precipitation from a solution of cadmium sulphate with zinc sticks.

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6. Sulphuric acid approximately 0.1N.

7. $KMnO_4$ 0.1N standardized against sodium oxalate. For sodium determinations this solution is diluted to 0.02N.

8. NaCl, C.P. Merck.

9. Uranyl acetate, C.P. Merck.

Procedure.—Into a 15 c.c. centrifuge tube with sharply pointed tip, there is measured 1 c.c. of the solution containing 0.2 to 0.04 mg. of sodium, followed by ½ c.c. of absolute alcohol and 3 c.c. of the freshly filtered uranyl zinc acetate reagent; the mixture is stirred until the precipitate appears to be uniformly distributed in the solution. After standing at room temperature for one to two hours, the contents are centrifuged at a high speed for ten minutes. The supernatant liquid is carefully removed by suction, and the tube is allowed to drain on filter paper. The mouth of the tube is wiped dry with a piece of filter paper and 3 c.c. of the glacial acetic acid saturated with the triple salt are delivered from a pipette in such a way as to rinse the whole of the inside of the tube. The precipitate is broken up with the same stirring rod and centrifuged for five minutes. The acetic acid is decanted off and the tube is again drained. Washing is repeated once with ether. To prevent evaporation of the reagent, the tube is covered with a rubber cap during standing and centrifuging. Blank determination of the reagents is made under similar conditions (four minutes of stirring were found to be necessary to bring about the precipitation of the triple salt).

The uranyl zinc sodium acetate is dissolved in 1 c.c. of NH_2SO_4 and reduced with a cadmium pencil. This is accomplished by immersing the tube in a water-bath for twenty minutes. Time required for stirring is thus saved; reduction, of course, also takes place fairly rapidly at room temperature. After cooling, the pencil is washed with 1 c.c. of N H_2SO_4, and the solution is titrated with $KMnO_4$ solution.

Results and Discussion.—In preliminary experiments, a study was made of the washing reagents which have been used by previous investigators em-

TABLE I

A COMPARISON OF WASH REAGENTS

SODIUM TAKEN MG.	0.02N $KMnO_4$ C.C.	SODIUM FOUND MG.	ERROR %	WASH REAGENT
0.200	2.67	0.205	+2.5	Acetic acid once
0.200	2.60	0.199	−0.5	
0.200	2.64	0.202	+1.0	
0.200	2.58	0.198	−1.0	Acetic acid and ether
0.200	2.60	0.199	−0.5	
0.200	2.58	0.198	−1.0	
0.200	2.61	0.200	0.0	Acetic acid, alcohol, and ether
0.200	2.56	0.196	−2.0	
0.200	2.57	0.199	−0.5	
0.200	2.64	0.202	+1.0	Alcohol twice, and ether
0.200	2.66	0.204	+2.0	
0.200	2.73	0.209	+4.5	

ploying the triple acetate method. The data in Table I, which were obtained in one day, indicate that with the centrifuge technic acetic acid followed by ether is a good wash-reagent.

TABLE II

DETERMINATION OF 0.20 TO 0.04 MG. OF SODIUM

SODIUM TAKEN MG.	0.02N $KMnO_4$ C.C.	SODIUM FOUND MG.	ERROR %	SODIUM TAKEN MG.	0.02N $KMnO_4$ C.C.	SODIUM FOUND MG.	ERROR %
0.200	2.69	0.206	+3.0	a 0.10	1.30	0.100	
0.200	2.69	0.206	+3.0	a 0.10	1.30	0.100	
0.200	2.68	0.205	+2.5	a 0.10	1.35	0.103	+3.0
0.200	2.58	0.198	−1.0	b 0.10	1.32	0.101	+1.0
0.200	2.60	0.199	−0.5	b 0.10	1.33	0.102	+2.0
0.200	2.58	0.198	−1.0	b 0.10	1.33	0.102	+2.0
0.200	2.62	0.201	+0.5	0.080	1.07	0.082	+2.5
0.200	2.64	0.202	+1.0	0.080	1.07	0.082	+2.5
0.200	2.65	0.203	+1.5	0.080	1.09	0.083	+3.8
0.150	1.97	0.151	+0.7	0.060	0.76	0.058	−3.3
0.150	1.91	0.146	−2.7	0.060	0.74	0.057	−5.0
0.150	1.94	0.149	−0.7	0.060	0.74	0.057	−5.0
0.100	1.25	0.096	−4.0	0.050	0.66	0.050	0.0
0.100	1.25	0.096	−4.0	0.050	0.67	0.051	+2.0
0.100	1.27	0.097	−3.0	0.050	0.64	0.049	−2.0
0.100	1.32	0.101	+1.0	0.040	0.49	0.038	−5.0
0.100	1.31	0.100	0.0	0.040	0.51	0.039	−2.5
0.100	1.32	0.101	+1.0	0.040	0.49	0.038	−5.0

a, 0.8 mg. of potassium and b, 0.4 mg. of potassium added to the sodium solution (1 c.c.).

TABLE III

TITRATION OF URANIUM, USING CADMIUM AS THE REDUCTANT

(A) JONES' REDUCTOR				(B) CADMIUM PENCIL IN CENTRIFUGE TUBE			
UO_2AC_2 IN N-H_2SO_4 TAKEN C.C.	0.01N $KMnO_4$ CALCD. C.C.	0.01N $KMnO_4$ USED C.C.	ERROR %	UO_2AC_2 IN N-H_2SO_4 TAKEN C.C.	0.02N $KMnO_4$ CALCD. C.C.	0.02N $KMnO_4$ USED C.C.	ERROR %
1/80 N 1.00	1.25	1.26	+0.8	1/80 N 1.00	0.625	0.63	+0.8
1/80 N 1.00	1.25	1.24	−0.8	1/80 N 1.00	0.625	0.63	+0.8
1/80 N 1.00	1.25	1.26	+0.8	1/80 N 1.00	0.625	0.62	−0.8
1/80 N 1.00	1.25	1.28	+2.4	1/80 N 1.00	0.625	0.62	−0.8
				1/80 N 1.00	0.625	*0.625	0.0
				1/80 N 1.00	0.625	*0.625	0.0
1/40 N 1.00	2.50	2.55	+2.0	1/40 N 1.00	1.25	1.23	−1.6
1/40 N 1.00	2.50	2.52	+0.8	1/40 N 1.00	1.25	1.25	0.0
1/40 N 1.00	2.50	2.51	+0.4	1/40 N 1.00	1.25	1.24	−0.8
1/40 N 1.00	2.50	2.55	+2.0	1/40 N 1.00	1.25	1.25	0.0
1/20 N 1.0	5.00	5.01	+0.2	1/20 N 1.00	2.50	2.49	−0.4
1/20 N 1.0	5.00	5.04	+0.8	1/20 N 1.00	2.50	2.47	−1.2
1/20 N 1.0	5.00	5.04	+0.8	1/20 N 1.00	2.50	2.47	−1.2
1/20 N 1.0	5.00	5.03	+0.6	1/20 N 1.00	2.50	2.50	0.0
1/10 N 1.0	10.00	10.05	+0.5	1/10 N 1.00	5.00	5.00	0.0
1/10 N 1.0	10.00	10.05	+0.5	1/10 N 1.00	5.00	4.96	−0.8
1/10 N 1.0	10.00	9.98	−0.2	1/10 N 1.00	5.00	4.97	−0.6
1/10 N 1.0	10.00	9.92	−0.8	1/10 N 1.00	5.00	4.97	−0.6

*At room temperature.

The determinations of the various quantities of sodium in Table II were made in triplicate. Blanks also in triplicate amounted to between 0.05 ± 0.01 to 0.17 ± 0.03 c.c. of 0.02n $KMnO_4$ in various experiments. The average value of the blanks in a given experiment was used in the calculation of the quantity of sodium in the sample; therefore the variation in the blanks is included in the error of determination.

Contrary to the results of Monk and Netter who used a different method, potassium in the amount present in striated muscle (Peters and Van Slyke) did not interfere with the determination of sodium by the alcoholic uranyl zinc acetate reagent described above (Table II).

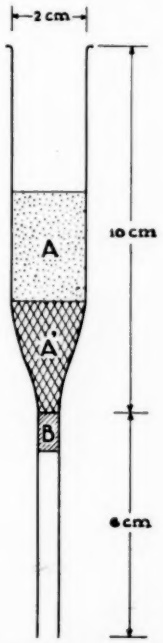

Fig. 1.—Jones' reductor for reducing $Ur.^{VI}$ to $Ur.^{IV}$ *A*, Loosely placed cadmium powder. *A*,' Tightly pressed cadmium powder. *B*, Filter paper.

TITRATION OF URANIUM WITH CADMIUM AS THE REDUCTANT

Zinc has long been used as the reductant for the volumetric determination of uranium. However, some of the quadrivalent uranium is invariably reduced further to the trivalent form; it is then necessary to pass air through the solution just long enough to oxidize the trivalent metal to the quadrivalent (Kolthoff and Lingane). Cadmium, being a milder reductant, was chosen in the present work. At the time of experiments, I was not aware of the work by Treadwell on the same subject. The results here reported in Table III agree well with those reported by him in his description of a macrotitration method of determining uranium provided that air is not excluded.

The data in Table III (*A*) were obtained with Jones' reductor. The acid solution to be reduced was made into a paste with the loosely placed cadmium

powder (Fig. 1) and allowed to stand for five minutes. It was filtered and washed three times with 2 c.c. portions of N H_2SO_4 and once with water. Each time the paste was well stirred before suction was applied.

The procedure for the determination of the uranyl sodium zinc acetate with cadmium pencil in a centrifuge tube was used to obtain the results in Table III (*B*). It is simple and as accurate as that employing the Jones' reductor.

SUMMARY

1. A simple and rapid microvolumetric method for the accurate determination of 0.20 to 0.04 mg. of sodium is described.

2. Sodium is precipitated in the presence of alcohol as uranyl zinc sodium acetate and the uranium, after reduction by cadmium, is determined by titration with $KMnO_4$ in the same centrifuge tube used for the precipitation of the triple salt.

3. Cadmium is shown to be a satisfactory reductant for the volumetric determination of uranium.

REFERENCES

1. Blanchetière, A.: Sur une méthode de dosage du sodium, Bull. Soc. chim. de France **33**: 807, 1923.
2. Barber, H. H., and Kolthoff, I. M.: A Specific Reagent for the Rapid Gravimetric Determination of Sodium, J. Am. Chem. Soc. **50**: 1625, 1928.
3. Caley, E. R., and Foulk, C. W.: A Gravimetric and Colorimetric Method for the Direct Determination of Sodium, J. Am. Chem. Soc. **51**: 1664, 1929.
4. Salit, P. W.: New Triple Acetate Method for Sodium Determinations in Biological Materials, J. Biol. Chem. **96**: 659, 1932.
5. Marenzi, A. D., and Gerschman, R.: Le microdosage du sodium du sérum ou du plasma, Compt. rend. Soc. de biol. **114**: 1212, 1933.
6. McCance, R. S., and Shipp, H. L.: Colorimetric Determination of Sodium, Biochem. J. **25**: 449, 1931.
7. Mond, R., and Netter, H.: Über die Regulation des Natriums durch den Muskel, Pflüger's Arch. f. d. ges. Physiol. **230**: 42, 1932.
8. Peters and Van Slyke: Quantitative Clinical Chemistry **2**: Baltimore, Md., Williams and Wilkins Co.
9. Kolthoff, I. M., and Lingane, J. J.: The Volumetric Determination of Uranium With Potassium Dichromate as Reagent and the Application of the Method to the Indirect Titration of Minute Quantities of Sodium, J. Am. Chem. Soc. **55**: 1871, 1933.
10. Treadwell, W. D.: Reduktionen mit Cadmium in der Massanalyse II, Helvetica Chimica Acta **5**: 732, 1922.

A MODIFICATION OF WRIGHT'S TECHNIC FOR THE DETERMINATION OF THE OPSONIC INDEX*

W. H. Crane, M.S., and J. L. Brakefield, Ph.D., Birmingham, Ala.

IT WAS found that the technic employed by Wright for the determination of the opsonic index was difficult to follow and required considerable time. It is believed that the outline given below will facilitate the determination.

The bacterial emulsion and the serums are prepared as described by Wright. The suspension of leucocytes follows the method by Wright except that 2 c.c. of blood are used rather than a few drops. Larger quantities appear to give better results. Rather than placing the three parts, namely, the suspension of leucocytes, the bacterial emulsion, and the serums, in a capillary tube to be sealed and incubated, the component parts are mixed thoroughly by drawing them into a white blood counting pipette, and then blowing them onto a hollow-ground slide. This mixing process is repeated several times. A hanging drop is prepared and incubated at 37° C. for fifteen minutes. Smears are then made from the drop.

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CONTROL OF MANGE IN LABORATORY DOGS†

F. W. Kinard, Ph.D., and J. van de Erve, M.D., Charleston, S. C.

MANY laboratories use dogs caught in the city streets. A large percentage of these dogs are mangy or else soon contract the disease from infected animals. For the last few years, this laboratory has been very successful in curing the disease in infected animals and in preventing its appearance in other animals, by the use of a low cost dip.

The United States Department of Agriculture[1] recognizes three sorts of dog mange. No attempt has been made to classify the cases among our dogs, but it is supposed that the sarcoptic form predominates.

The dip is prepared in a barrel by mixing 1.0 part of lime-sulphur concentrate and 8.0 parts of water. The animal is immersed to its ears and then released in the pen. The same dip is used repeatedly.

The animal is dipped when it is delivered to the laboratory, then again in about two weeks, and thereafter at intervals of about six weeks.

Lime-sulphur concentrate is a liquid spray material used extensively by fruit growers.

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The United States Department of Agriculture[2] lists several of the chemical compounds present in the spray, as calcium pentasulphide, calcium tetrasulphide, calcium thiosulphate, sulphur, and calcium sulphite. The concentrate generally retails for about twenty-five cents a gallon.*

SUMMARY

A low cost, efficient dip is described. It has been of great value in curing and preventing mange in laboratory dogs.

REFERENCES

1. U. S. Dept. Agric., Dept. Cir. No. 338.
2. U. S. Dept. Agric., Farmers Bull. No. 1285.

*Epting Distributing Company, Leesville, S. C.

A NEW MICROREACTION FOR THE SERODIAGNOSIS OF SYPHILIS†

An Adaptation of the Kahn Standard Antigen

Arturo R. Casilli, M.D., F.A.C.P., Elizabeth, N. J.

ELIMINATING the hemolytic system simplified the serodiagnosis of syphilis by substituting the relatively simple flocculation and precipitation tests for the complicated complement fixation method. Although flocculation tests had been attempted previously, it was the events of the Great War which stimulated German workers to reinvestigate the problem, with the result that Sachs-Georgi gave to the world one of the first practical applications of flocculation in the serodiagnosis of syphilis. The results of this test were impressive but not completely convincing, consequently many experimenters attempted to remove its imperfections. It was R. L. Kahn, however, who produced a notably simple flocculation test, which has successfully withstood critical clinical analysis. The Kahn test is generally accepted to be sensitive and specific; nevertheless, the originator and numerous later investigators have aimed to increase its sensitivity.

Recent work has led to the development of various slide tests, which have gained favor with progressive serologists. The chief advantage of the slide test is the use of a smaller amount of serum in producing the microreaction. On the other hand, it must be noted that these modifications have not, on the whole, yielded more accurate results or simplified technical procedures. Besides, the antigen used for the microreaction is usually reinforced with tincture of gums or resins, thereby endangering specificity. Perhaps Kline's slide test is the most promising of its type, as it gives results equaling, and according to its author and others, surpassing those of the Kahn test. In my opinion, however, the Kline method increases technical difficulties by requiring an especially prepared antigen and particular equipment.

†Received for publication, November 6, 1935.

That the antigen, necessary for the serodiagnosis of syphilis, is theoretically nonspecific, but practically specific, is well recognized by all serologists. The reinforcement of heart lipoidal extracts with cholesterin is known to render them more sensitive. Kline in his brilliant work has probably carried the saturation of lipoids with cholesterin to the maximum degree. However, at this point, I wish to emphasize that when 6 mg. of cholesterin are added to every 1 c.c. of lipoidal extract, cholesterinization is sufficient and safe for practical purposes, giving specific and sensitive results. The method which I propose is based on the recognition of this fact. The advantages are: (1) That it requires no special equipment, (2) that it is extremely simple, (3) that the readings are clear-cut, (4) that the reaction is immediate, and (5) that there is no danger of producing false precipitation or readings in the hands of the average technician. I believe that, in specificity and sensitivity, this method, at least, equals the Kahn test tube method.

TABLE 1

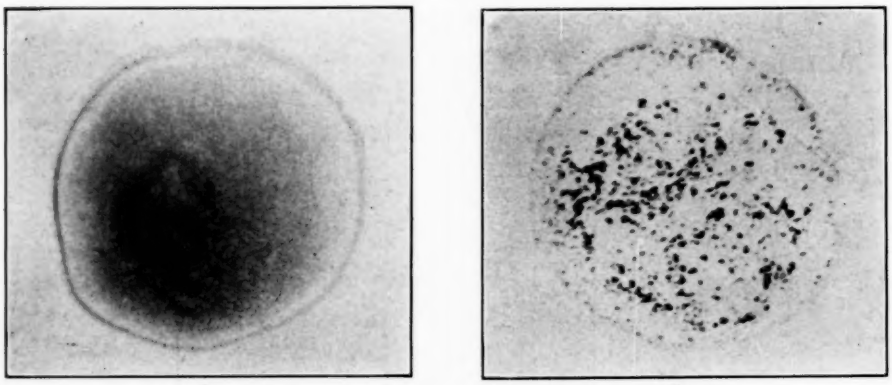
SLIDE COMPARISON TABLE

NUMBER	KAHN	WASSERMANN	SLIDE
	Complete Agreement		
68	++++	++++	++++
1	++	++	++
104	----	----	----
Total 173			
	Agreement With Kahn—Disagreement With Wass.		
3	++++	+++	++++
4	++	----	++
2	+	----	+
1	+	A.C.	+
Total 10			
	Agreement With Wass.—Disagreement With Kahn		
1	+	----	----
Total 1			
	Disagreement With Both Wass. and Kahn		
1	++	++	++++
1	++	----	++++
1	+	----	++++
1	++	----	+++
1	+	----	+++
1	++++	++++	++
4	+	----	++
1	+	+	----
Total 11			
Grand total 195			

Agreement of slide with Kahn	98.97%
Agreement of slide with Wassermann	90.77%
Agreement of Kahn with Wassermann	90.77%

Method.—The antigen is prepared and titrated exactly according to the instructions given by Kahn. After the antigen salt mixture precipitate has been standing for one-half hour, although ten minutes will be sufficient, the mixture is centrifuged at high speed for approximately ten minutes. This last step constitutes the first modification. The idea, however, is not new, as some years ago I read of a similar procedure in, I believe, an abstract by the *Journal*

of the American Medical Association, but unfortunately I cannot remember the name of the author, nor does a search of medical literature as far back as 1909 reveal his identity. The clear, supernatant, alcoholic salt solution is carefully decanted off. A clean piece of soft, white cloth is inserted into the tube, down to but not touching the mush at the bottom, so as to absorb as much as possible of the alcoholic salt solution remaining. This mush is a yellowish white, lipoidal, cholesterinized mixture, and it is this substance which is used in the test. This separation of the antigen mush from the alcoholic salt solution constitutes the second and really significant modification. Now place on three clean slides, previously labelled known positive, known negative, and unknown, 0.1 c.c. of the inactivated serum corresponding to the label. A smaller amount of inactivated serum can be used with equally good results. One loopful of antigen mush is then added to each of the serums and with the same loop emulsified until no visible particles remain. A thin, platinum wire loop,



A. *B.*

Fig. 1.—*A*, negative; *B*, four-plus.

0.5 mm. in diameter is used, and is sterilized in the flame and allowed to cool before each loopful is taken. This procedure may be continued for as many unknown serums as desired, but the writer feels that five unknown serums is the maximum number advisable.

Results.—Clumping almost immediately follows emulsification in the positive serums; the negative ones remain milky and homogeneous. For weaker positive serums a minute may elapse before clumping is observed. The results are read with the naked eye, and best observed with reflected light from below. The observation should not exceed five minutes and clumping is facilitated by occasional gentle tilting of the slide back and forth. Reading should be made as strongly positive, weakly positive, and negative, although with added experience more quantitative readings are not, as a rule, difficult. The positive and negative reactions are clearly seen in Fig. 1. When cold a very fine granular appearance may be noted in the negative serums, but this is quite different from the large flakes of positive clumping.

Comment.—It is conceded that the success of the Kahn reaction is due to the concentration of antigen in preparation and titer, and the serum-antigen ratios. In the slide test proposed, there is maximum concentration by utilizing

the mush while the serum-antigen ratios remain constant. Important, perhaps, is the fact that with the removal of practically all of the alcoholic salt solution, the reaction is almost entirely colloidal in nature. I realize that 200 comparison tests of the standard three tube Kahn, Wassermann, and my slide test are not a sufficient number from which to draw final conclusions, but I feel that this number is sufficient to justify the publication of this method.

CONCLUSIONS

1. A simple adaptation of the Kahn antigen for slide agglutination test is presented.
2. The results obtained are specific and sensitive.
3. The readings are simple and rapid.
4. No special equipment or especially prepared antigen are needed.
5. The serums must be inactivated.
6. Alcoholic salt solution from the first precipitate is almost entirely removed.
7. The test is made with a mush of heart lipoids and cholesterin.

I wish to express my thanks to Dr. R. L. Kahn for kindly supplying me with antigen prepared by his laboratory, and to Messrs. James R. Chester and Edward Connelly for aid in performing the tests.

REFERENCES

Kahn, R. L.: Serum Diagnosis of Syphilis by Precipitation; Governing Principles, Procedure, and Clinical Application of the Kahn Precipitation Test, 8°, Baltimore, 1925. The Kahn Test; a Practical Guide, 8°, Baltimore, 1928. Relation Between Serum and Antigen in Precipitation Reaction for Syphilis Proposed by Author, Proc. Soc. Exper. Biol. & Med. **19**: 295, 1921. A Simple Quantitative Precipitation Reaction for Syphilis, Arch. Dermat. & Syph. **5**: 570 and 734; **6**: 332, 1922. The Method of Titrating Antigen for Kahn Precipitation Test, Proc. Soc. Exper. Biol. & Med. **20**: 489, 1922. Rapid Precipitation Phase of the Kahn Test for Syphilis With New Method for Indicating Results, J. A. M. A. **81**: 89, 1923. Improved Procedure of Kahn Precipitation Test for Syphilis. Papers Mich. Acad. Sc. Arts & Lett., 1923, N. Y. **3**: 319, 1924.

Kline, B. T.: Microscopic Slide Precipitation Test for the Diagnosis and Exclusion of Syphilis, J. LAB. & CLIN. MED. **16**: 186, 1920. Microscopic Slide Precipitation Test With Unheated Serum, J. LAB. & CLIN. MED. **15**: 686, 1930.

Meinicke, E.: Information on Syphilis by Flocculation Reactions, Deutsche med. Wchnschr. **46**: 13, 1920. Micro-reaction on Syphilis, Monatschr. f. Kinderh. **29**: 481, 1924. On the Technique of My Lues-Reaction, Deutsche med. Wchnschr. **43**: 660, 1917. On the Third Modification of My Lues-Reaction, München. med. Wchnschr. **66**: 932, 1919. On the Technique of My Turbidity Reaction for Syphilis (MTR), Klin. Wchnschr. **3**: 361, 1924. The Meinicke Micro-Reaction for Syphilis, Med. Klin. **21**: 132, 1925.

Sachs, H.: Demonstration of a New Method for Sero-Diagnosis of Syphilis (Flocculation Reaction According to Sachs and Georgi), München. med. Wchnschr. **66**: 84, 1919.

Sachs, H., and Georgi, W.: On the Sero-Diagnosis of Syphilis by Means of Flocculation With Cholesterinized Extracts, Med. Klin. **14**: 805, 1918.

Casilli, A. R.: New Technique for the Flocculation Test of Syphilis, J. A. M. A. **80**: 1068, 1923.

A STAINING TECHNIC FOR BLOOD IN SPINAL FLUID*

Method to Differentiate Between Recent and Old Hemorrhage

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IN THE course of a series of experiments on rats dealing with increased intracranial pressure and hypertension, we occasionally encountered, on cisternal puncture, a bloody cerebrospinal fluid. It seemed desirable to know whether this blood was the result of a fresh hemorrhage, or of one that had occurred a day or more previously. As a survey of the literature failed to reveal a method for making such a differentiation, we set about to devise one. Because of its clinical applicability, which we are not in a position to utilize, we are reporting our results simply to describe the method.

Experimental Method.—White rats were used. A healthy animal was anesthetized with ether, the transverse ridge at the junction of the frontal and parietal bones, anterior to the posterior fourth of the cranium, located and, using this as a landmark, a cutting edge needle was thrust through the skull, into and just through one of the lateral sinuses, a distance of about 3 mm. After withdrawal of the needle, there is free external bleeding, and spinal fluid obtained by cisternal puncture is grossly bloody. Striking the lateral sinus is fairly easy with practice, especially if a skull cap be removed from a dead animal for orientation. At first, cisternal tap was done immediately after the lateral sinus puncture, approximately 0.03 c.c. of bloody spinal fluid withdrawn, dropped on a slide, smeared, and let dry. Later it was thought better to wait for thirty to sixty minutes before doing the cisternal tap, as it seemed easier to procure fluid then. However, the smears made during this short interval were found to be identical in character. A second cisternal puncture was done on most animals at the end of twenty-four hours; in a few animals this was omitted, and they were tapped at forty-eight hours. It was found that in the beginning when the puncture of the lateral sinus was done rather clumsily some bleeding might continue for many hours, hence the necessity of the forty-eight-hour tap. However, as we gained skill and experience, we found that the bleeding from the sinus puncture soon stopped and the twenty-four-hour smear showed the changes seen also in the forty-eight-hour smear, so the longer period became unnecessary. Lately, all our animals were simply tapped one hour after the lateral sinus puncture, and again after twenty-four hours.

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As an in vitro control, the following procedure was followed in two persons who were having lumbar puncture done for some other purpose. To 2 c.c. of sterile spinal fluid, 0.05 c.c. of blood obtained aseptically from an arm vein were added. The tube was shaken to insure mixing, and a drop or two removed, smeared on a slide, and let dry. The tube, still presumably sterile, was then incubated at 37° C. for twenty-four hours, and more smears made in the manner previously described.

Staining Technic.—In the preliminary work a number of stains were used, including Wright's, Giemsa, cresol blue, and Sudan III, as well as attempts at agglutination with antiserums. However, staining with Sudan III was the only procedure which gave us the information desired. Its original selection was suggested on the assumption that the lipoid content of the red cell is rich as contrasted with the spinal fluid, and that in time some alteration of lipoid distribution might occur. The correctness or incorrectness of the assumption has not been further tested.

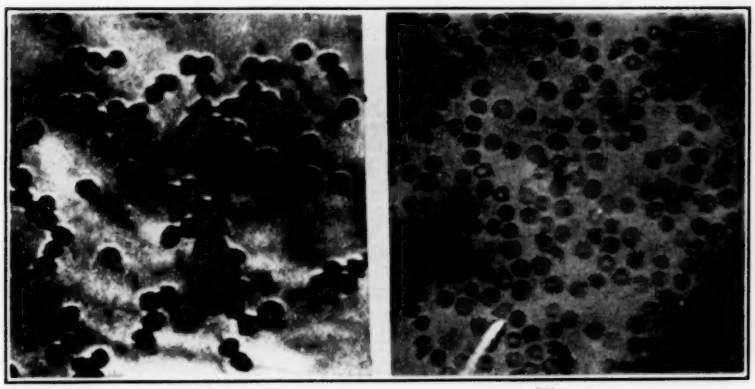

Fig. 1. Fig. 2.

Fig. 1.—Untouched photograph showing red cells in spinal fluid one hour after subarachnoid hemorrhage. Stained by Sudan III.

Fig. 2.—Untouched photograph showing red cells in spinal fluid twenty-four hours after subarachnoid hemorrhage. Note lighter stain and relatively clear central areas seen in many cells. Stained by Sudan III.

The smear of spinal fluid containing blood was permitted to dry thoroughly in the air, requiring fifteen to thirty minutes. We tried many fixatives, including heat, formaldehyde vapor, 10 per cent formalin, and methyl alcohol, but discarded them all as unnecessary. Simple drying appeared to suffice, but if a fixative is desired, passing the smear through a flame as for a blood preparation, being careful not to overheat, appears to be unobjectionable. The slide was then immersed for twenty minutes in 50 per cent alcohol saturated with Sudan III. Then it was thoroughly rinsed in several changes of distilled water, requiring three to four minutes, and then blotted on filter paper (never permitted to dry in air!). It was then covered with glycerin (which is not a lipoid solvent), mounted with a cover slip, and examined. The stain fades in a day or so, and preparations should be examined fresh. Attempts to secure permanence by ringing with asphalt are disappointing.

Discussion of Results.—Thirty rats were used. In smears made within an hour of the hemorrhage into the cerebrospinal fluid, the red cells appear as

round homogeneously staining pale yellow disks (see Fig. 1). Although in a single focus a few may appear to have pale centers, it usually may be ascertained by focusing that this appearance is due simply to the normal central depression. In smears made twenty-four hours after a properly done lateral sinus puncture, the appearance is quite different. Most of the cells present central unstained areas, making the peripheral stained portions present somewhat the appearance of rings (see Fig. 2). Occasionally a few of the cells may still show a homogeneous stain, but they are relatively few. The difference between the appearance of the two smears, when freshly stained, is quite striking.

A few smears (not included in the series of thirty) have been made at intervals from two to six hours after the hemorrhage. Such smears show more of the older forms, with pale centers, but most of the cells still show a uniform yellow stain.

Two smears made from human spinal fluid, to which had been added some of the individual's own blood, showed essentially the same phenomena. The cells in the fresh smear were pale yellow and homogeneous, while those twenty-four-hours old showed stain-free centers and ring-appearing peripheries.

In five rats and 3 human beings, we have performed the following procedure. One tenth of a cubic centimeter of blood is added to 3 c.c. of physiologic saline, agitated, and smears made in the usual way. These cells show the homogeneous stain characteristic of the fresh spinal fluid smear. It is suggested that this procedure might be used in a patient in whom the time of subarachnoid hemorrhage was unknown, in order to obtain a smear which could be accepted as identical with one containing fresh blood, which could then be compared with the smear actually obtained from his spinal fluid.

SUMMARY

Smears of spinal fluid of rats were obtained one hour and twenty-four hours after subarachnoid hemorrhage. A staining technic, using Sudan III, is described by which smears obtained at these two intervals stain differently. The red cells of the fresh smear stain a homogeneous yellow, while those twenty-four hours old, in general, stain less distinctly and have unstained centers. The same staining difference was found in fresh and twenty-four-hour smears by an in vitro method, using human spinal fluid to which blood from the same person had been added. It was also found that fresh blood mixed with normal saline presents in the stained smear the same appearance as fresh blood in spinal fluid, a procedure which may form a standard for comparison in a clinical case.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

TRYPANOSOMIASIS, Animal, A Stable Alcoholic Antigen for the Diagnosis of, by Complement Deviation, Zottner, G. Compt. rend. Soc. de Biol. 115: 19, 1934.

Rats infected with *T. equiperdum* or *T. marocanum* are bled into 1.5 per cent sodium citrate solution at the height of the infection.

The subsequent steps are as follows:

1. Sediment the red cells by slow centrifugation in a hand machine.
2. Remove the plasma.
3. Centrifuge plasma at high speed to throw down the trypanosomes (white or rosy deposit).
4. Wash the deposit once with distilled water (to lyse any red cells) and twice with normal saline.
5. After third washing remove supernatant fluid as completely as possible and add a little acetone to the deposit.
6. Evaporate the acetone in the incubator.
7. Grind up the deposit in a mortar with a few drops of absolute alcohol.
8. Prepare a suspension of 0.1 gram of dried trypanosomes in 10 c.c. of absolute alcohol.
9. Place suspension in sealed amber glass bottles and incubate fifteen days at 40° C. shaking thoroughly each day.

The antigen is now ready for titration and use.

MONONUCLEOSIS, Infectious, Some Cytologic and Serologic Aspects of, Stuart, C. A., Burgess, A. M., Lawson, H. A., and Wellman, H. E. Arch. Int. Med. 54: 199, 1934.

A brief presentation of the clinical and cytologic aspects of infectious mononucleosis, based on a study of 28 sporadic cases of the disease, and a more extended report of serologic investigations of the disease are submitted.

The morphologic characteristics of the predominant blood cell at the height of the disease suggest that it is an immature lymphocyte.

Supravital staining methods employed in one case support this view.

Evidence of amitosis of lymphocytes in the circulating blood stream similar to that noted in lymphatic leucemia is presented.

It is demonstrated that the agglutination of sheep cells by normal human serum is greatly influenced by the concentration of cells used and the temperature at which the serum is incubated.

The agglutination of sheep cells by normal human serum at low temperatures is reversible at 37.5° C. in most cases.

Serum from patients with infectious mononucleosis agglutinates sheep cells equally well at 5° C. and at 37.5° C.

Certain changes in the technic of the serologic test for infectious mononucleosis are proposed (see below).

It is pointed out that the serologic test without confirmatory clinical and cytologic findings may result in false diagnoses.

Certain facts must be explained before the heterophile nature of the antibody in mononucleosis can be unreservedly accepted.

The authors propose the following changes in the conduct of the agglutination test:

Paul and Bunnell used 0.5 c.c. of the serum dilution, 0.5 c.c. of a 2 per cent sheep cell

suspension and 1 c.c. of saline solution. The total volume of 2 c.c. results in such a column of liquid in the usual 100 by 10 mm. tubes that a satisfactory reading can be made by only placing the finger over the end of the tube and tilting it 3 or 5 times. In routine work there is a certain element of danger in this method since in the same rack a technician may have agglutination tests for pathogenic organisms, and it is not improbable that on occasions the wrong tube may be inverted with the finger over the end. The extra 1 c.c. of saline solution plays no rôle in the test and should be omitted. The same concentration of cells can be obtained by adding 0.5 c.c. of a 1 per cent suspension. In order to insure uniform sheep cell suspensions for the test all suspensions should be prepared from 2 c.c. of concentrated washed cells which have been centrifugated in 8 c.c. of saline solution for five minutes at a speed of approximately 2,200 revolutions a minute. A final reading should be made after four hours of incubation at 37.5° C., since incubation for two hours at this temperature is not sufficient to give complete agglutination in any dilution, regardless of the final titer. If this is not convenient the tests, as soon as completed, may be put in the ice box overnight to be read the next morning after incubation for two hours at 37.5° C. with occasional shaking. In this way the often confusing cold agglutination is dispersed, making the diagnosis far more certain. The authors are of the opinion that a serum which agglutinates sheep cells by this procedure in a dilution of 1:80 may be classed as suggestive. But it should be remembered that the agglutinins do not appear in the blood stream in any significant concentration until several days after the first appearance of symptoms; therefore a suggestive serum should not be made the basis of a negative diagnosis until further tests have been made. With substantiating clinical and cytologic pictures, serums agglutinating in dilutions of 1:320 or more may well be considered positive.

STAINING METHODS, Rapid, Maneval, W. E. Science 80: 292, 1934.

The author reports upon methods for negative staining of bacteria. In addition to the usual 1 per cent aqueous nigrosin, 2 per cent Congo red may be used which may be turned to blue by the addition of 1 drop of 1 per cent HCl in 95 per cent ethyl alcohol. Other dyes may be added to the nigrosin solution to give varying colored backgrounds and, in some cases, to produce light staining of the organisms.

The most satisfactory are: rose bengal, magdala red, and cotton blue in 1 per cent solution added to the nigrosin solution in the proportion of 30 drops of 1 per cent aqueous solution to 40 c.c. of nigrosin solution, after which 4 drops of concentrated HCl are added.

When these combinations are used they may be fixed to the slide by covering with a drop of 2 per cent aqueous ferric chloride solution which, after one minute, is washed off with water.

The following method is recommended for the demonstration of mitotic figures:

Stain:	Distilled water	30 c.c.
	10 per cent alcoholic basic fuchsin	10 c.c.
	Anilin oil 1 part and 95 per cent alcohol 3 parts mixed	5 c.c.
	Acetic acid 4 per cent	1 c.c.

Mix in order given, filter twice, and again before using. Solution best used after standing 3 to 6 days and remains good 3 to 5 weeks. Filter when necessary.

Method:
1. Remove paraffin.
2. Treat with absolute, 95 per cent, 70 per cent, 50 per cent, and 30 per cent alcohols on slide, adding 2 or 3 drops of each in succession, allowing to act for twenty to thirty seconds, and drain.
3. Water.
4. Stain for three to five minutes.
5. Wash in 3 or 4 changes of water.
6. Dehydrate in alcohols to 95 per cent containing a trace of HCl.
7. 95 per cent to absolute alcohol.

8. Counterstain with a 1:3 mixture of 0.1 per cent solutions Orange G and light green in clove oil.
9. Wash in absolute alcohol.
10. Clear in xylol and mount in balsam.

Anilin blue in 90 per cent alcohol is also a good counterstain.

AGRANULOCYTIC ANGINA, The Relation of Amidopyrin and Allied Drugs to the Etiology of, Jackson, H., Jr. Am. J. M. Sc. **188:** 482, 1934.

In 26 per cent of the cases studied the disease followed the administration of amidopyrine or allied drugs. That in these instances the disease may have been actually caused by the therapy is possible, perhaps probable.

In another 30 per cent of the cases a critical examination of the evidence shows that in spite of the fact that these drugs were taken in considerable quantities they definitely have no causative relation to the disease.

Of these patients, 44 per cent received no drugs of this type whatsoever, yet their clinical and hematologic pictures were similar in every respect.

RABIES, Influence of Virus on the Agglutination of Proteus X-19 Organisms, D'Aunoy, R., and Fine, A. J. Trop. Med. & Hyg., March, 1934.

The authors have not been able to find any reference in the literature to the possibility of typhus fever being mistaken for rabies on the basis of agglutinative phenomena. On the contrary, it is quite well established that the Weil-Felix reaction occurs in no disease but typhus. Felix states: "It is the unanimous opinion of those who have used the test extensively that an X-19 agglutination of diagnostic significance does not occur in any disease except typhus." Fletcher and Lesslar, after extensive experience, state: "The Weil-Felix reaction has not been found to occur in any disease except typhus."

The present investigation substantiates in every detail the view of absolute absence of serologic relationship between typhus and rabic infections, be the latter of the fixed or street virus types.

1. The blood serum of human beings suffering from rabies shows no agglutination content for proteus X-19 organisms.

2. Induced fixed and street virus rabies in animals do not cause the production of agglutinins for proteus X-19 organisms.

3. Prophylactic inoculations in the human of killed fixed rabic virus emulsions do not result in the production in any demonstrable amounts of agglutinins for proteus X-19 organisms.

DEXTROSE TOLERANCE: The One-Hour Two-Dose Test, Exton, W. G., and Rose, A. R. Am. J. Clin. Path. **4:** 381, 1934.

Dissolve 100 gm. of glucose in about 650 c.c. of water. This solution is then flavored with lemon and divided into two equal doses containing 50 gm. of glucose in about a 15 per cent solution, which are served cold and are easy to drink within a minute. Keep at hand three containers with preservative against glucolysis for the blood specimens and three containers for the urine specimens, making it a point when collecting these to have subjects empty the bladder as completely as possible. The following steps are taken after a fast, preferably overnight:

1. Collect blood and urine (A) samples and give the first dose of glucose, allowing one to two minutes for its ingestion.

2. Thirty minutes after ingestion of the glucose, collect blood (B) sample and give the second dose of glucose, allowing one to two minutes for its ingestion.

3. Thirty minutes after the ingestion of the second dose of glucose, collect blood and urine (C) samples. The third urine container is given the subject for a sample of the urine next voided whenever a (post) sample is desired.

The typical criteria of normal responses to the one-hour two-dose test are: (1) a fasting blood sugar within the normal limits of the particular blood sugar method employed; (2) a rise in blood sugar which does not exceed 75 mg. in the thirty-minute sample; (3) the blood sugar in the sixty-minute sample is less, the same, or does not exceed the thirty-minute sample by more than 5 mg., and (4) all urine samples are negative to Benedict's test.

The criteria for determining diabetes in the one-hour two-dose test are a more or less steep rise of not less than 10 mg. of blood sugar in the sixty-minute sample following the second dose of glucose and the relation of blood and urine sugar values to the severity of the disease.

The criteria of alimentary glycosuria are, therefore: a sugar-free urine after fasting with sugar in the final urine and blood sugars that follow the normal curve even when the level is higher than normal.

Besides its greater convenience all the available evidence indicates that results of this new method are more specific and reliable than the methods now in general use.

IMPETIGO CONTAGIOSA, New Auxiliary Treatment for, Hollander, L., and Hecht, J. J. Am. J. Dis Child. **48**: 269, 1934.

The use of metaphen 1:500 in collodion has been introduced for the treatment of pyogenic infections of the skin. The results obtained were so satisfactory that this treatment is presented as an auxiliary one for impetigo contagiosa, ecthyma, and infectious eczematoid dermatitis (pustular eczema and impetiginous eczema).

The advantages of the use of the metaphen-collodion preparation are:

1. The ease of application.

2. The impervious character of the dressings, easily confining the infection, so that auto-inoculability and contagion become less likely and thus the disease does not necessitate the exclusion of school children from their classroom activities, except in unusually severe cases.

Method: The skin surface surrounding the infected area is washed with soap and water and dried thoroughly with absorbent cotton. The lesion is painted with several layers of metaphen 1:500 in collodion, which is permitted to dry layer by layer.

In twenty-four hours the easily removable layers of the metaphen-collodion mixture are removed with a tissue forceps. The adherent part is left on, and the mixture is reapplied in several layers. The procedure is repeated on the third day. On the fourth day all of the metaphen-collodion preparation, which by that time has curled up at the edges, is removed, and with it the underlying crustation. If the underlying skin is dry, light anointing with 2 per cent ammoniated mercury is carried out. If, however, the surface is still moist, the metaphen-collodion mixture is applied for another period of three days.

PROTOZOA, An Improvement in Staining Technic For, Sargent, D. L. Stain Technol. **11**: 49, 1936.

A portion of the fecal material to be examined is suspended in a 0.6 per cent salt solution; the suspension should be of a consistency so that one drop will make a satisfactory microscope mount under a cover glass. To ten parts of this suspension, in a test tube, is added one part of the stain which is prepared as follows:

10 parts of distilled water
6 parts of a suspension of colloidal iodine (Chandler) containing 4 per cent iodine—20 per cent iodine suspensoid, Merck
1 part of a 10 per cent water solution of anilin red, Merck (eosin yellowish)

Technicians will find, because iodine in the form of colloidal iodine is readily released to the organisms, that the use of this material is far superior to Lugol's solution in carrying out the technic for staining intestinal protozoa in the study of fresh mount preparations. Not only are organisms more deeply stained with iodine but by eosin as well, even when employed in high dilutions.

CEREBROSPINAL FLUIDS, On the Differentiation of, Robinson, F. H., and Miller, B. N. Am. J. M. Sc. **191**: 538, 1936.

The benzidin reaction is positive in cerebrospinal fluid containing a hemoglobin dilution of 1 to 50,000 (100 erythrocytes per c.mm.), but not to a dilution of 1 in 100,000 (50 erythrocytes per c.mm.).

The benzidin reaction on the supernatant fluid after centrifuging at high speed is of value in differentiating hemorrhage into the cerebrospinal fluid at the time of puncture from subarachnoid hemorrhage.

An extramedullary tumor compressing the spinal cord in its growth has been reproduced experimentally in dogs with a resulting Nonne-Froin syndrome in the cerebrospinal fluid below the level of the compression.

The yellow spinal fluid of cord compression gives a negative benzidin reaction.

B. TUBERCULOSIS, An Inexpensive Synthetic Medium for, Wong, S., and Weinzirl, J. Am. Rev. Tubere. 33: 577, 1936.

MODIFIED LONG AND SEIBERT'S MEDIUM

Mallic acid	3.00 gm.
Ammonium hydroxide (10 per cent solution)	12.00 c.c.
Ammonium citrate	5.00 gm.
Monopotassium acid phosphate	6.00 gm.
Sodium carbonate (anhydrous)	2.00 gm.
Sodium chloride	2.00 gm.
Magnesium sulphate	1.00 gm.
Ferric ammonium citrate	0.05 gm.
Glycerol	50.00 c.c.
Distilled water, to make	1,000.00 c.c.
Glucose solution to be added after sterilization	10.00 gm.

Ingredients may be dissolved by means of heat if necessary. The initial pH should be 6.8 to 7.0, the final pH about 7.0. The medium was autoclaved at 20 pounds' pressure for fifteen minutes. A 20 per cent C.P. dextrose solution is sterilized in test tubes to facilitate introduction into the flasks. The amount placed in each tube is such that when added to the medium the final concentration is approximately 1 per cent. If it becomes necessary to readjust the pH after autoclaving the medium must be titrated to pH 7.4 and autoclaved again at 20 pounds' pressure for fifteen minutes. Apparently reautoclaving does not alter the usefulness of the medium.

PNEUMONIA, Pneumococcus Type III—An Analysis of 500 Cases, Cecil, R. L., Plummer, N., and McCall, M. Am. J. M. Sc. **191**: 305, 1936.

Pneumococcus Type III ranks third as an exciting agent in lobar pneumonia, causing 11.8 per cent of all pneumococcal pneumonias. It is surpassed only by pneumococcus Type I and Type II in respect to prevalence.

The incidence of pneumococcus Type III pneumonia increases with age. In the present series pneumococcus Type III was responsible for 32.8 per cent of all pneumococcal pneumonias that occurred after the age of sixty.

Pneumococcus Type III pneumonia is relatively commoner in women than in men. The incidence rate for women is 18.2 per cent, as compared with 11.6 per cent for men. This is in sharp contrast to Type I and Type II infections, where the incidence per cent for the two latter types is considerably higher for males than for females.

Pneumococcus Type III pneumonia is very prone to occur in individuals who are already the victim of some chronic disease. In the present series of 500 cases, 49.9 per cent of the patients were afflicted with some chronic malady. This association of chronic disease with pneumonia was much higher in Type III than in Type I or Type II infections.

Bacteremia was noted in 29.4 per cent of the Type III patients who were subjected to blood cultures.

The death rate for the entire series of 500 cases of Type III pneumonia was 42.2 per cent, a figure which agrees closely with that reported by other investigators. In Bellevue Hospital, however, Type III pneumonia ranks second in severity, being surpassed by pneumococcus Type II pneumonia with a death rate of 48.8 per cent.

The death rate for Type III pneumonia was influenced by a number of factors. In bacteremic cases it was 85.4 per cent. The death rate is much affected by age and the incidence of chronic systemic disease. In patients under forty years the death rate was approximately 20 per cent; over sixty years, 65.4 per cent. In patients without systemic disease the death rate was 32.1 per cent; with systemic disease, 53.6 per cent.

There is no satisfactory serum therapy for pneumococcus Type III pneumonia at the present time. The most promising outlook with respect to a specific treatment for this infection is the enzyme of Avery, which destroys the specific carbohydrate in the capsule of pneumococcus Type III, and which in the case of animals infected experimentally with lethal doses of Type III pneumococcus, has shown distinctly curative properties.

Correspondence

Note on "Improved Procedures for Estimating Bismuth in Body Fluids and Tissues"

Dear Sir:

Under this title, a paper, published by Lahman, Richardson and Hanzlik in this JOURNAL (**21**: 95, 1935) contains a table (p. 97) which is in need of some clarification. Therefore, the following additional information is submitted:

The data in the second column of the table entitled, "Colorimeter Readings," were obtained by comparison with a bismuth standard of 0.001 mg. set at 90 on the colorimeter.

In the fourth column, entitled "Colorimeter Reading," the data were obtained after correction for 0.0009 mg. Bi, the blank value. The standard for comparison was generally the next higher amount and the actual readings in descending order were 97, 64, 77, 84, 20.5, 22.9, and 87, before correction for 0.0009 mg.

P. J. HANZLIK, M.D.

July 3, 1936
Stanford University School of Medicine
San Francisco, Calif.